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(54) Title: METHOD FOR THE PURIFICATIOIN OF INTACT, CORRECTLY-FOLDED INSULIN-LIKE GROWTH FACTOR-1

(57) Abstract

Method for the recovery and purification of intact, correctly-folded, monomeric insulin-like growth factor-1 peptide from large volumes of IGF-1-containing medium are described, comprising a series of adsorption-desorption steps employing a combination of cation exchange and hydrophobic interaction adsorbents. Product IGF-1 peptide is highly purified and suitable for use in a variety of clinical applications.

METHOD FOR THE PURIFICATION OF INTACT, CORRECTLY-FOLDED INSULIN-LIKE GROWTH FACTOR-1

5   This invention relates to purification methods.
In a particular aspect, this invention relates to the
purification of insulin-like growth factor-1 peptides
from fluid medium containing same. In one aspect, the
present invention relates to methods for purification of
10   insulin-like growth factor-1 peptides produced by
recombinant techniques. In another aspect, the present
invention relates to methods for the purification of
insulin-like growth factor-1 peptides produced by yeast
cells transformed with at least one copy of a DNA
15   sequence encoding an insulin-like growth factor-1
peptide.

BACKGROUND OF THE INVENTION
          Insulin-like growth factor-1 (IGF-1) is a
20   polypeptide of 70 amino acids with a molecular weight of
7648 daltons. This single chain protein has three
intrachain disulfide bridges. These disulfide bonds,
along with numerous hydrogen bonds and hydrophilic
interactions, maintain the compact tertiary structure of
25   this molecule. However, it has been shown that, upon
reduction and reoxidation, IGF-1 can refold in a variety
of ways, forming as many as 15 monomeric configurations
[see Meng, et al., J. Chrom. 443:183 (1988)].
Consequently, attempts to produce large quantities of
30   this peptide can lead to the formation of a complicated
mixture of product forms which must be purified for
further use.
          Insulin-like growth factor-1 belongs to a
heterogeneous family of peptides which share some of the
35   biological and chemical properties of insulin, but which
are antigenically distinct from insulin. Currently
available experimental evidence suggests that IGF-1

-2-

promotes growth by mediating the effects of growth
hormone.   Thus, such processes as skeletal growth, cell
replication and other growth related processes are
affected by IGF-1 levels. Physiological concentrations
5   of IGF-1 have been shown to be influenced by such
conditions as thyroid disease, diabetes and malnutrition
[see Preece, in Horm. Blood, 4: 108 (1983)].

IGF-1 has also been shown to act
synergistically with other growth factors, for example,
10   in accelerating the healing of soft and mesenchymal
tissue wounds [see Lynch et al., in J. Clin.
Periodontol., 16: 545 (1989) and Lynch et al., in Proc.
Natl. Acad. Sci. USA, 84: 7696 (1987)], and in enhancing
the growth of mammalian cells in serum-free tissue
15   culture medium [see Burleigh and Meng, in American
Biotech. Lab., 4: 48 (1986)].

Considering the many clinical and research
applications of IGF-1, a ready supply of IGF-1 will be of
great value to the medical and biotechnology fields.
20   Since isolation from natural sources is technically
difficult, expensive, and time consuming, recent efforts
have centered on the development of efficient recombinant
methods for the production of IGF-1.

The methylotrophic yeast Pichia pastoris has
25   recently been developed as an improved host for the
production of recombinant products. Recombinant Pichia
pastoris strains have been shown to be capable of
secreting certain recombinant proteins in the gram per
liter range. In addition, such strains have been shown
30   to be capable of adapting to fed batch or continuous
cultivation fermentation conditions. Moreover, such
strains have an extremely stable recombinant phenotype
and are capable of maintaining high yields of the desired
recombinant expression product over several orders of
35   fermentation scale. Indeed, Brierley, et al., in
copending application United States Serial No. 578,728,
filed September 4, 1990, have recently shown that P.

-4-

Brierley et al. also describe in co-owned
international application No. PCT/US91/06452, which is a
continuation-in-part of U.S. Serial No. 578,728 filed
with the Patent Cooperation Treaty on September 4, 1991,
5   production of IGF-1 in P. pastoris strains deficient in
proteolytic activities that can degrade the recombinant
product to yield aberrant forms such as nicked IGF-1.
The use of protease-deficient strains of P. pastoris as
hosts for recombinant expression of heterologous proteins
10   susceptible to degradation by P. pastoris proteases is
described in U.S. Serial No. 07/678,916 filed April 1,
1991.   The disclosure of these co-pending applications is
hereby incorporated by reference in their entirety.
Fermentations of IGF-1-producing P. pastoris strains
15   deficient in proteolytic activity yielded 50-100% more
authentic IGF-1 and 30% less nicked IGF-1 than similar
fermentations of IGF-1-producing P. pastoris strains that
were not deficient in proteolytic activity. Although
purification of authentic IGF-1 from the broth of a
20   P. pastoris strain deficient in proteolytic activity
might be facilitated by the lower amounts of nicked IGF-1
in the broth, a separation process capable of
distinguishing between the various forms of IGF-1 is
still required for such purification.

25

SUMMARY OF THE INVENTION
In accordance with the present invention, we
have developed an efficient method for the recovery and
purification of IGF-1 peptides from fluid medium
30   containing same. The invention method involves
successive selective adsorption-desorption steps on a
combination of cation exchange and hydrophobic
interaction chromatography matrix materials and may
preferably include gel filtration chromatography.   In
35   this way, about 30-50% recovery of substantially
purified, intact, correctly-folded, monomeric IGF-1 can
be accomplished in the presence of various other forms of

-3-

pastoris is an excellent host for the recombinant
production of IGF-1. The disclosure of this copending
application is hereby incorporated by reference in its
entirety. In view of the availability of medium
5   containing high levels of recombinantly produced IGF-1,
there is needed an efficient means for the recovery and
purification of IGF-1 from such medium.

        Recombinantly produced IGF-1 frequently
consists of a mixture of several different forms of
10   IGF-1, i.e., intact, monomeric, correctly-folded material
(also referred to herein as authentic IGF-1), as well as
various aberrant forms, such as, misfolded material
(i.e., having improperly formed disulfide bonds), nicked
material (i.e., wherein one or more of the peptide bonds
15   of the amino acid backbone have been broken, but the
molecular weight of the resulting species is
substantially the same as that of intact material, since
the nicked material has the same number of amino acid
residues as intact material, and the fragments of nicked
20   material are held together by disulfide bonds), cleaved
material (e.g., wherein one or more peptide bonds are
broken so that two fragments of lower molecular weight,
relative to intact material, are produced; or peptide
lacking one or more amino acid residues relative to
25   intact material), multimeric forms (i.e., dimers,
trimers, etc., wherein disulfide bonds are formed between
two or more different IGF-1 monomer chains), and so on.
Due to the substantial similarity of the various forms of
IGF-1, the purification of recombinantly produced
30   material presents a difficult technical challenge. Not
only does such purification require the separation of
IGF-1 peptides from the other peptides produced during
fermentation, in addition, a separation is required which
is selective enough to distinguish between the various
35   forms of IGF-1 which may be present.

contacting said matrix first with in the range f about 1
up to 10 volumes, relative to the volume of matrix, of a
buffer system having a sufficiently low conductivity so
as to displace aberrant IGF-1 peptides from said matrix,

5   without displacing significant quantities of the intact,
monomeric, correctly-folded form of adsorbed IGF-1 from
said first hydrophobic interaction chromatography matrix,
followed by contacting said matrix with in the range of
about 1 up to 10 volumes, relative to the volume of

10   matrix, of a buffer system having an elevated pH, wherein
said elevated pH is sufficiently high so as to displace
substantially all of the remaining adsorbed forms of
IGF-1 from said matrix.

        In accordance with another of its aspects, the

15   present invention entails a method for the purification
of monomeric intact, correctly-folded insulin-like growth
factor-1 peptide (IGF-1) from medium containing IGF-1
peptides, said method comprising:

        (a)   contacting said medium with a sufficient

20   quantity of first cation exchange material under
conditions suitable to adsorb at least about 95% of total
IGF-1 from said medium,

        (b)   eluting the adsorbed IGF-1 from the
IGF-1-containing cation exchange material of step (a) by

25   contacting said cation exchange material with a
sufficient quantity of a solvent system which has a
sufficiently high pH or ionic strength so as to displace
substantially all of said IGF-1 from said cation exchange
material,

30   (c)   contacting the IGF-1-containing fractions
of the eluate of step (b), in a suitable solvent system,
with a sufficient quantity of a first hydrophobic
interaction chromatography matrix under conditions
suitable to adsorb in the range of about 95 up to 100% of

35   said IGF-1 from said eluate,

        (d)   eluting the adsorbed IGF-1 from said first
hydrophobic interaction chromatography matrix by

-5-

IGF-1 which are initially present in the crude IGF-1
containing medium.

BRIEF DESCRIPTION OF THE FIGURES

    Figure 1 is an elution profile for elution of
various IGF-1 species from the first hydrophobic
interaction chromatography matrix.
    Figure 2 is an elution profile for elution of
various IGF-1 species from the second cation exchange
matrix.

DETAILED DESCRIPTION OF THE INVENTION

    In one aspect, the present invention entails a
method for the purification of monomeric intact,
correctly-folded insulin-like growth factor-1 peptide
(IGF-1) from medium containing IGF-1 peptides, said
method comprising:
    (a)   contacting said medium with a sufficient
quantity of first cation exchange material under
conditions suitable to adsorb at least about 95% of total
IGF-1 from said medium,
    (b)   eluting the adsorbed IGF-1 from the
IGF-1-containing cation exchange material of step (a) by
contacting said cation exchange material with a
sufficient quantity of a solvent system which has a
sufficiently high pH or ionic strength so as to displace
substantially all of said IGF-1 from said cation exchange
material,
    (c)   contacting the IGF-1-containing fractions
of the eluate of step (b), in a suitable solvent system,
with a sufficient quantity of a first hydrophobic
interaction chromatography matrix under conditions
suitable to adsorb in the range of about 95 up to 100% of
said IGF-1 from said eluate,
    (d)   luting the adsorbed IGF-1 from said first
hydroph bic interaction chromatography matrix by

-8-

quantity of a solvent system which has a sufficiently
high pH or ionic strength so as to displace substantially
all of said IGF-1 from said cation exchange material,

(c) contacting the IGF-1-containing fractions
5 of the eluate of step (b), in a suitable solvent system,
with a sufficient quantity of a first hydrophobic
interaction chromatography matrix under conditions
suitable to adsorb in the range of about 95 up to 100% of
said IGF-1 from said eluate,

10 (d) eluting the adsorbed IGF-1 from said first
hydrophobic interaction chromatography matrix by
contacting said matrix first with in the range of about 1
up to 10 volumes, relative to the volume of resin, of a
buffer system having a sufficiently low conductivity so
15 as to displace some of the aberrant IGF-1 peptides from
said matrix, without displacing significant quantities of
the intact, monomeric, correctly-folded form of adsorbed
IGF-1 from said first hydrophobic interaction
chromatography matrix, followed by contacting said matrix
20 with in the range of about 1 up to 10 volumes, relative
to the volume of matrix, of a buffer system having an
elevated pH, wherein said elevated pH is sufficiently
high so as to displace substantially all of the remaining
adsorbed forms of IGF-1 from said matrix,

25 (e) contacting the fractions eluted according
to step (d) employing said buffer system having an
elevated pH which contain, as the predominant form of
IGF-1, intact, monomeric, correctly-folded IGF-1, wherein
said contacting is carried out with a sufficient quantity
30 of a second cation exchange matrix under conditions
suitable to adsorb in the range of about 95 up to 100% of
total IGF-1 from said eluate,

(f) eluting the adsorbed IGF-1 from said second
cation exchange matrix by contacting said matrix with at
35 least one volume, relativ to the volum of matrix, of a
buffer system having a sufficient ionic strength so as to

-7-

contacting said matrix first with in the range of about 1
up to 10 volumes, relative to the volume of matrix, of a
buffer system having a sufficiently low conductivity so
as to displace aberrant IGF-1 peptides from said matrix,
5   without displacing significant quantities of the intact,
monomeric, correctly-folded form of adsorbed IGF-1 from
said first hydrophobic interaction chromatography matrix,
followed by contacting said matrix with in the range of
about 1 up to 10 volumes, relative to the volume of
10   matrix, of a buffer system having an elevated pH, wherein
said elevated pH is sufficiently high so as to displace
substantially all of the remaining adsorbed forms of
IGF-1 from said matrix;

               (e) contacting the intact, monomeric,
15   correctly-folded IGF-1-containing fractions of the eluate
from step (d), in a suitable solvent system, with a
sufficient quantity of a gel filtration chromatography
matrix having suitable pore size to effect resolution of
the intact, monomeric correctly-folded form of IGF-1 from
20   substantially all multimeric forms of
IGF-1; and

               (f) eluting said gel filtration chromatography
matrix with a sufficient quantity of an eluent so as to
cause the intact, monomeric correctly-folded form of IGF-
25   1 to be resolved from said multimeric forms of IGF-1.

               In accordance with the present invention, there
is provided a method for the purification of monomeric,
intact, correctly-folded insulin-like growth factor-1
peptide (IGF-1) from medium containing IGF-1 peptides,
30   said method comprising:

               (a)   contacting said medium with a sufficient
quantity of first cation exchange matrix under conditions
suitable to adsorb at least about 95% of total IGF-1 from
said medium,

35   (b)   eluting the adsorbed IGF-1 from the
IGF-1-containing cation exchange matrix of step (a) by
contacting said cation exchange matrix with a sufficient

containing IGF-1 peptides, wherein said medium containing
IGF-1 is the substantially cell-fr e fermentation broth
from a high cell density yeast fermentation operation,
and wherein said yeast are transformed with at least one

5   DNA fragment comprising, in the direction of
transcription, the following DNA sequences:

(i)   a promoter region of a methanol responsive
gene of P. pastoris,

10   (ii) a DNA sequence encoding a polypeptide
consisting of:

(a)   the S. cerevisiae alpha mating factor
(AMF) pre-pro sequence, including a
processing site selected from the

15   group consisting of lys-arg; and lys-
arg-(glu-ala)$_x$, wherein x is an
integer between 1 and about 3, and

(b)   an insulin-like growth factor-1
(IGF-1) peptide; and

20   (iii)   a transcription terminator functional
in P. pastoris,

wherein said DNA sequences are operationally associated
with one another for transcription of the sequences
encoding said polypeptide, said method comprising:

25   (a) contacting said medium with a
sufficient quantity of a sulfylpropylated cation exchange
media under conditions suitable to adsorb at least about
95% of said IGF-1 from said medium; wherein at least 0.03
liters, per gram of IGF-1 in said medium, of said cation

30   exchange material are employed; and wherein said
contacting is carried out at a temperature in the range
of about 2 up to 30°C, and wherein, optionally, the IGF-
1-containing media may be diluted prior to contacting
with the cation exchange material with a low conductivity

35   buffered medium having the same pH as the medium us d to
equilibrate the cation exchange material,

-9-

differentially displace substantially all of the IGF-1
peptides from said matrix,

      (g)   contacting the intact, monomeric,
correctly-folded IGF-1-containing fractions of the eluate

5 from step (f), in a suitable solvent system, with either
(1) a sufficient quantity of a second hydrophobic
interaction chromatography matrix under conditions
suitable to adsorb in the range of about 95 up to 100% of
all forms of IGF-1 from said eluate, or (2) a sufficient

10 quantity of a gel filtration matrix having suitable pore
size to effect resolution of the intact, monomeric,
correctly folded form of IGF-1 from substantially all
multimeric forms of IGF-1 and,

      (h)(1) after step (g)(1), eluting adsorbed

15 IGF-1 from said second hydrophobic interaction
chromatography matrix by contacting said matrix first
with in the range of about 1 up to 10 volumes, relative
to the volume of matrix, of a buffer system having a
sufficiently low conductivity so as to displace

20 substantially all forms of IGF-1 other than the intact,
monomeric, correctly-folded form of IGF-1 from said
matrix, without displacing significant quantities of the
intact, monomeric, correctly-folded form of adsorbed IGF-
1, followed by contacting said matrix with in the range

25 of about 1 up to 10 volumes, relative to the volume of
matrix, of a buffer system having a sufficiently high pH
so as to displace substantially all of the remaining
adsorbed IGF-1 from said matrix or (2) after step (g)(2),
eluting said gel filtration chromatography matrix with a

30 sufficient quantity of elution buffer as to cause the
intact, monomeric, correctly folded form of IGF-1 to be
resolved from said multimeric forms of IGF-1.

      In accordance with a specific embodiment of the
present invention, there is provided a method for the

35 purification of monomeric, intact, correctly-folded
insulin-like growth factor-1 (IGF-1) peptide from medium

-12-

the ammonium sulfate concentration of the eluate in the range of about 0.2 up to 2 $\underline{M}$, and the pH of the diluted eluate about 4.5,

    (e) contacting the product of step (d) with a sufficient quantity of a first hydrophobic interaction chromatography matrix under conditions suitable to adsorb in the range of about 95 up to 100% of said IGF-1 from said eluate, wherein said first hydrophobic interaction chromatography matrix is a butyl-substituted, poly(methacrylate)-supported hydrophobic interaction chromatography matrix; wherein at least about 0.05 liters, per gram of IGF-1 in said medium, of said hydrophobic interaction chromatography matrix are employed; and wherein said contacting is carried out at a temperature in the range of about 20 up to 25°C,

    (f) eluting the adsorbed IGF-1 from said first hydrophobic interaction chromatography matrix by contacting said matrix:

    (1) first with a quantity of a linear salt gradient of a buffered solution having a pH of about 4.5, or, preferably, an initial pH of about 5.0 and a final pH of about 4.0, sufficient to produce a substantially ammonium sulfate-free eluate, and,

    (2) preferably, with a buffered solution having a pH of about 4.0, then

    (3) with (i) a quantity of a linear gradient of a substantially ammonium sulfate-free buffered solution having an initial pH of about 4.0-4.5 sufficient to raise the pH of said eluate up to about 6.5-7.5, or (ii) a quantity of a buffered solution having a pH of about 6.5 to 7.5, wherein step (f)(3)(ii) is preferably carried out after step (f)(2).,

-11-

(b) contacting the IGF-1-containing cation
exchange material with at least about 2 volumes, per
volume of said cation exchange material, of a 0.02 $\underline{M}$
acetic acid solution, followed by either (1) about four
5   volumes of a 0.02M sodium acetate solution having a pH of
5 and containing 0.2M sodium chloride or (2) at least
four volumes of a 0.05M sodium acetate solution having a
pH of 5 and then at least four volumes of a 0.05M sodium
acetate solution having a pH of 5.5 or (3) about four
10   volumes of a 0.05M sodium acetate solution, pH 5.5, then
about four volumes of a solution containing 0.05M NaCl in
50 mM sodium acetate pH 5.5 and then about four volumes
of a solution containing 0.1M NaCl in 50 mM sodium
acetate, pH 5.5,
15   (c)(1) eluting the adsorbed IGF-1 from
said IGF-1-containing cation exchange matrix material of
step (b)(1) by contacting said matrix material of step
(b)(1) with a sufficient quantity of a solvent system
comprising a 0.02 $\underline{M}$ sodium acetate solution having a pH
20   of 5.5 and containing 1.0 $\underline{M}$ sodium chloride, or (2)
eluting the adsorbed IGF-1 from said IGF-1-containing
cation exchange matrix material of step (b).(2) by
contacting said matrix material of step (b)(2) with about
8 volumes, per volume of said cation exchange matrix, of
25   a linear gradient solution comprising 0 to 0.5M NaCl in
50 mM sodium acetate, pH 5.5 or (3) eluting the adsorbed
IGF-1 from said IGF-1-containing cation exchange matrix
material of step (b)(3) by contacting said matrix
material of step (b)(3) with about 8 volumes of a
30   solution containing 0.3M NaCl in 50 mM sodium acetate, pH
5.5,
                (d) contacting the eluate of step (c)(1)
or those portions of the eluate of step (c)(2) or (c)(3)
that contain, as the predominant form of IGF-1, intact,
35   monomeric correctly folded IGF-1 with a sufficient volume
of a buffered ammonium sulfate-containing solution,
having a pH between about 4.0 and 7.0, so as to render

-14-

1, intact, monomeric, correctly-folded IGF-1, wherein
said contacting is carried out with a sufficient quantity
of a second cation exchange matrix material and under
conditions suitable to adsorb in the range of about 95 up
5   to 100% of said IGF-1 from said eluate, wherein said
second cation exchange matrix is a sulfylmethylated or
sulfylpropylated matrix; wherein at least about 0.05
liters, per gram of IGF-1 in said medium, of said matrix
are employed; and wherein said contacting is carried out
10   preferably at a temperature in the range of about 20 up
to 25°C,

(j) contacting the IGF-1-containing cation
exchange matrix material with at least one, up to about
five volumes, per volume of said cation exchange matrix
15   material, of a 0.05 $\underline{M}$ sodium acetate solution, pH 4.5,
and, preferably with 1-5 volumes of a 0.05 M sodium
acetate solution, pH 5.5,

(k) eluting the adsorbed IGF-1 from said
second cation exchange matrix material by contacting said
20   matrix material with at least five volumes, relative to
the volume of matrix, of a sodium chloride gradient,
which is provided by combining, as a linear gradient, a
first solvent system and a second solvent system;

wherein said first solvent system
25   comprises a 0.05 $\underline{M}$ sodium acetate solution, pH 5.5, and

wherein said second solvent system
comprises a 0.05 $\underline{M}$ sodium acetate/0.3 $\underline{M}$ sodium chloride
solution, pH 5.5,

(l) after step (k), either (1) diluting
30   the intact, monomeric, correctly-folded IGF-1-containing
fractions of the eluate of step (k) with at least one
volume of a buffered ammonium sulfate-containing
solution, pH 4.0-7.0, so as to render the ammonium
sulfate concentration of the eluate in the range of about
35   0.2 up to 2.0 $\underline{M}$ and the pH of the diluted eluate about
4.5, and contacting the diluted eluate with a sufficient
quantity of a second hydrophobic interaction

-13-

(g)   optionally, contacting at least a portion of the eluate fractions obtained in step (f)(3)(i) with a sufficient additional quantity of said first hydrophobic interaction chromatography matrix under

5   conditions suitable to adsorb in the range of about 95 up to 100% of residual quantities of IGF-1 from said eluate, wherein said first hydrophobic interaction chromatography matrix is a butyl-substituted hydrophobic interaction chromatography matrix; wherein at least about 0.05

10   liters, per gram of IGF-1 in said medium, of said first hydrophobic interaction chromatography matrix are employed; and wherein said contacting is carried out at a temperature in the range of about 20 up to 25°C,

(h)   if optional step (g) was conducted,

15   eluting the adsorbed IGF-1 from said first hydrophobic interaction chromatography matrix by contacting said matrix:

(1)   first with a quantity of a linear salt gradient of a buffered solution having a

20   pH of about 4.5, or, preferably, an initial pH of about 5.0 and a final pH of about 4.0, sufficient to produce a substantially ammonium sulfate-free eluate, and,

25   (2) preferably, with a buffered solution having a pH of about 4.0, then

(3) with (i) a quantity of a linear gradient of a substantially ammonium sulfate-free buffered solution having an initial pH of

30   about 4.0-4.5 sufficient to raise the pH of said eluate up to about 6.5-7.5, or (ii) a quantity of a buffered solution having a pH of about 6.5 to 7.5,

(i) contacting those portions of the

35   eluate from (f)(3)(i) or step (f)(3)(ii) or, optionally, the combined eluates from (f)(3)(i) and step (h)(3)(i) or (h)(3)(ii) which contain, as the predominant form of IGF-

intact monomeric correctly folded form of IGF-1 to be resolved from said multimeric forms of IGF-1.

The term "insulin-like growth factor-1" or "IGF-1 peptide" or simply "IGF-1", as used throughout the specification and in the claims, refers to the various forms of recombinantly produced IGF-1 (i.e., intact, monomeric, correctly-folded IGF-1, plus nicked, cleaved, multimeric and misfolded forms). As employed herein, the term "intact, monomeric, correctly-folded IGF-1" refers to forms of IGF-1 having substantially the same 70 amino acid sequence and tertiary structure as native IGF-1 (see Rotwein, et al., J. Biol. Chem. 261:4828-4832 (1986)), as well as biologically active analogs and derivatives thereof.

The first step in the invention purification process is to contact insulin-like growth factor-1-containing medium with a sufficient quantity of a first cation exchange matrix under conditions suitable to adsorb at least about 95% of the total IGF-1 from the medium.

Contacting of the insulin-like growth factor-1-containing medium with the first cation exchange matrix can be carried out in a variety of ways. For example, the first cation exchange matrix can be contained in a column through which the insulin-like growth factor-1-containing medium is percolated. Alternatively, the first cation exchange matrix can be contained in a closed vessel into which the insulin-like growth factor-1-containing medium is introduced, followed by stirring for a sufficient period of time to allow contacting of the matrix and fluid medium to occur, followed by decanting of the IGF-1-depleted medium from the IGF-1-containing first cation exchange matrix. As another alternative, the first cation exchange matrix can be added to the vessel containing the IGF-1-containing medium, followed by removal of IGF-1-depleted medium from the IGF-1-containing first cation exchange matrix.

-15-

chromatography matrix under conditions suitable to adsorb
in the range of about 95 up to 100% of said IGF-1 from
said eluate; wherein said second hydrophobic interaction
chromatography matrix is a butyl-substituted,
5   poly(methacrylate)-supported hydrophobic interaction
chromatography matrix; wherein at least about 0.05
liters, per gram of IGF-1 in said medium, of said second
hydrophobic interaction chromatography matrix are
employed; and wherein said contacting is carried out at a
10   temperature in the range of about 20 up to 25°C, or (2)
concentrating the intact, monomeric, correctly-folded
IGF-1-containing fractions of the eluate of step (k) and
then contacting the concentrated material with a
sufficient quantity of a size exclusion media having
15   suitable pore size to effect resolution of the intact,
monomeric correctly folded form of IGF-1 from
substantially all multimeric forms of IGF-1 and
                (m)(1) after step (l)(1) eluting the
adsorbed IGF-1 from said second hydrophobic interaction
20   chromatography matrix by contacting said matrix:
                (i)   first with a quantity of a linear salt
gradient of a buffered solution having a pH of about 4.5,
or preferably an initial pH of about 5.0 and a final pH
of about 4.0, sufficient to produce a substantially
25   ammonium sulfate-free eluate, and
                (ii)   preferably with a buffered solution
having a pH of about 4.0, then
                (iii)   with either (1) a quantity of a linear
gradient of a substantially ammonium sulfate-free
30   buffered solution having an initial pH of about 4.0-4.5
sufficient to raise the pH of the eluate up to about 6.5-
7.5 or (2) a quantity of a buffered solution having a pH
of about 6.5 to about 7.5 or
                (m)(2) after step (l)(2), eluting said size
35   exclusion matrix with 1-2 volumes of 0.05-0.1 M ammonium
acetate, pH 6, or 0.2M acetic acid so as to cause the

-18-

carried out for a tim in the range of about 0.01 up to 1
hour, or longer, and at a temperature in the range of
about 2 up to 30°C; with temperature in the range of
about 22 up to 25°C being preferred.

5   Once IGF-1-containing broth has been maintained
in contact with the first cation exchange matrix for a
period of time sufficient for IGF-1 to adsorb to the
matrix material, it is desirable to remove the
IGF-1-depleted medium from contact with the IGF-1-rich

10   first cation exchange matrix. This can be accomplished
in a variety of ways such as, for example, filtration,
decantation, centrifugation, and the like. This
contacting and separation can readily be accomplished in
one operational step by passing the medium containing

15   IGF-1 through a column of the first cation exchange
matrix, wherein the column is equipped with a retaining
means (e.g., a screen, support plate with holes, or the
like), so as to retain the first cation exchange matrix
in the column, yet allow fluid medium to pass

20   therethrough. In this way, the IGF-1-depleted broth is
allowed to merely flow through the first cation exchange
matrix. In carrying out column chromatography in
accordance with the present invention, a column bed
height greater than about 10 cm is desirable, with a bed

25   height of approximately 20 cm being especially preferred.
Once substantially all of the IGF-1 has been
adsorbed onto the first cation exchange matrix, and prior
to elution of the IGF-1 therefrom, it is desirable to
contact the IGF-1-containing matrix with in the range of

30   about 1 up to 10 volumes, relative to the volume of the
first cation exchange matrix, of a dilute, weak acid; and
thereafter, the matrix is further contacted with
additional volumes of dilute, weak acid at a higher ionic
strength (or at a higher ionic strength and pH) than the

35   initial wash. This optional wash procedure serves to
remove impurities which are not as tightly bound to the
first cati n exchange matrix as is the IGF-1. Exemplary

-17-

Cation exchange matrix materials contemplated for use in the first contacting step of th present invention are well known in the art. Such exchange matrices include strong cation exchange matrices capable

5   of having a high flow rate, i.e., having a high degree of strength so as to withstand high pressures, and, optionally, possessing a macroporous structure, and in addition, are capable of binding IGF-1 over a wide pH range. Exemplary strong cation exchange matrices include

10 carboxymethylated and sulfonated cation exchange matrices. Matrix materials such as cellulose, polystyrene, dextrans, agarose, cross-linked agarose, and the like, can be used for the preparation of cation exchange matrices. Presently preferred cation exchange

15 matrices for use in this first contacting step are sulfylpropylated matrices.

While not essential, it is frequently desirable to activate cation exchange matrices prior to contacting with IGF-1-containing medium. Such activation serves to

20 condition the matrix for the IGF-1-containing medium which follows, and improves the efficiency of the matrix for adsorption of IGF-1 peptides and the separation of such peptides from other components in the medium. A typical activation procedure involves sequential contact

25 of the cation exchange matrix with several column volumes of a dilute, weak acid (e.g., 2-5 volumes of 0.2 $\underline{M}$ acetic acid), followed by several additional column volumes of a more dilute weak acid solution (e.g., 2-10 volumes of a 0.02 $\underline{M}$ acetic acid solution).

30 The quantity of first cation exchange matrix material employed in the practice of the present invention can vary widely. Typically, at least about 0.03ℓ, up to 1 liter of matrix, per gram of IGF-1 contained in the medium, will be employed.

35 Contacting of IGF-1-containing medium with the first cation xchang matrix can be carried out under a variety of conditions. Typically such contacting is

-20-

about 1 M sodium chloride, (2) a linear gradient solution
comprising 0-0.5 M NaCl in 50 mM sodium acetate, pH 5.5
or (3) a 0.05 M sodium acetate solution containing 0.3 M
NaCl, pH 5.5. Where the elution step is carried out with
5   a 0.05 M sodium acetate solution, pH 5.5, containing 0.3
M NaCl (i.e., solvent system (3) above), it is
particularly preferred to use the wash system consisting
of 20 mM acetic acid followed by 50 mM sodium acetate, pH
5.5, followed by 0.1 M NaCl in 50 mM sodium acetate, pH
10 5.5 prior to the elution step.

        The quantity of the eluting solvent system
employed can vary widely. Typically, in the range of
about 3-10 volumes of the solvent system, per volume of
first cation exchange matrix, will be employed.

15   Elution of IGF-1 from the IGF-1-containing
cation exchange matrix can be carried out under a variety
of conditions. Typically, a temperature in the range of
about 2 up to 30°C will be employed. Typically, elution
time will be relatively short, falling in the range of
20 about 0.01 up to 1.0 hour, although longer or shorter
times can also be employed.

        Partially purified IGF-1-containing medium
which has been eluted from the first cation exchange
matrix is then contacted with a sufficient quantity of a
25 first hydrophobic interaction chromatography matrix
under conditions suitable to adsorb in the range of 95%
up to 100% of the IGF-1 from the IGF-1-containing medium.
While such contacting can be carried out in both batch
and continuous modes, it is presently preferred to
30 contain the matrix material in a column, and to pass the
IGF-1-containing medium therethrough. It is preferred to
use a sufficient amount of matrix material to give a
column bed height of about 20 cm.

        Prior to contacting the eluate of the first
35 cation exchange matrix with the first hydrophobic
interaction chromatography matrix, the initial eluate is
typically treated with a sufficient volume of a buffered

-19-

dilute, weak acid solutions include approximately 0.02
molar ac tic acid solutions or phosphoric acid solutions.
Presently preferred wash systems include 20 mM acetic
acid followed by 20 mM sodium acetate, pH 5, containing

5   0.2 M NaCl or 20 mM acetic acid followed by 50 mM sodium
acetate, pH 5, followed by 50 mM sodium acetate, pH 5.5,
or a wash system consisting of: 20 mM acetic acid
followed by 50 mM sodium acetate, pH 5.5, followed by
0.1 M NaCl in 50 mM sodium acetate, pH 5.5.

10   Once substantially all of the IGF-1 has been
adsorbed onto the first cation exchange matrix and
optionally rinsed as described above, IGF-1 is then
eluted from the IGF-1-containing matrix by contacting the
matrix with a sufficient quantity of a solvent system

15   which has a sufficiently high pH or ionic strength so as
to displace substantially all of the IGF-1 from the
matrix. A solvent system which has a sufficiently high
pH or ionic strength to accomplish the desired elution is
a solvent system which has a higher ionic strength than

20   the aqueous medium with which the matrix material is
equilibrated, or, alternatively, has a pH of greater than
the aqueous medium with which the matrix material is
equilibrated. Elution is accomplished by increasing the
pH or ionic strength of the solvent system. A solvent

25   system which has a "sufficiently high pH or ionic
strength" to accomplish the desired elution is one
wherein the pH or ionic strength has been sufficiently
altered so as to substantially increase the partitioning
of IGF-1 peptides into the mobile phase from the

30   stationary phase.
               Solvent systems contemplated for use in this
elution step include dilute buffered solutions of a weak
acid, at a pH of about 5.5, and containing about a 0.2-1M
concentration of an ionic salt, e.g., sodium chloride,

35   and the like. Presently preferred solvent systems for
us in this elution step are. (1) a 0.02 M sodium acetate
solution having a pH of about 5.5, further containing

-22-

preferred hydrophobic interaction chromatography matrix
for use in the practice of the present invention is a
butyl-substituted, poly(methacrylate) hydrophobic
interaction chromatography matrix (e.g., TSK butyl
5   Toyopearl-650M matrix).

        Prior to use, hydrophobic interaction
chromatography matrix can be activated, or after use,
hydrophobic interaction chromatography matrix can be
regenerated, employing the following sequential wash
10   procedure:
                in the range of 1-10 column volumes of water,
                in the range of 3-10 column volumes of a 0.5 M
sodium hydroxide solution,
                in the range of 1-10 column volumes of water
15   in the range of 3-10 column volumes of a 50%
aqueous methanol solution, and finally
                in the range of 1-10 column volumes of water,
and thereafter, the column is equilibrated with in the
range of 5-10 column volumes of a salt-containing
20   acetate/phosphate buffer having a pH of about 4.5,
preferably 5.0.
        Quantities of first hydrophobic interaction
chromatography matrix employed in the practice of the
present invention can vary widely. Typically, in the
25   range of about 0.05 up to 1 liter of matrix per gram of
IGF-1 in the medium being treated will be employed.
        Contacting of partially purified
IGF-1-containing broth with the first hydrophobic
interaction chromatography matrix can be carried out
30   under a variety of conditions. Typically, such
contacting is carried out for a time in the range of
about 0.1 up to 30 minutes and a temperature in the range
of about 15 up to 30°C; with temperatures in the range of
about 20 up to 25°C being preferred.
35   Once substantially all of the IGF-1 has been
adsorbed by the first hydrophobic interaction
chromatography matrix, the IGF-1 is eluted from the first

-21-

salt-containing solution, pH 4.0-7.0, preferably 4.5-5.0,
so as to render the salt concentration of the diluted
eluate in the range of about 0.4 up to 1.0 M, preferably
0.6 M, and the pH of the diluted eluate about 4.5.   By

5   increasing the salt content of the medium, the binding
affinity of IGF-1 for the hydrophobic interaction
chromatography matrix is enhanced.   Salts contemplated
for such use are those salts which improve the
hydrophobic interaction of IGF-1 and the hydrophobic

10   interaction chromatography matrix, e.g., sodium sulfate,
potassium sulfate, ammonium sulfate, potassium phosphate,
sodium acetate, ammonium acetate, sodium chloride, sodium
citrate, and the like.   Broadly, the salt content
employed will fall in the range of about 0.2 up to 2.0 M;

15   with a salt content of about 0.4 up to 1 M being
presently preferred.   An especially preferred salt is
ammonium sulfate, at a concentration of about 0.4-0.8 M.

Hydrophobic interaction chromatography matrix
materials contemplated for use in this next contacting of

20   the present invention are alkyl- or aryl-substituted
hydrophobic interaction chromatography matrices.
Hydrophobic interactions are a phenomenon of great
biological significance.   They are one of the main forces
that stabilize the three-dimensional structure of

25   proteins.   Hydrophobicity is the repulsion between a
nonpolar compound and a polar environment such as water.
Since the structure of water about a hydrophobic compound
creates hydrophobic interactions, if one changes the
structure of water by dissolving salts therein, the

30   hydrophobic interactions are affected.   Exemplary
matrices include butyl-, octyl-, or phenyl-substituted
hydrophobic interaction chromatography matrices.
Supports contemplated for use as hydrophobic interaction
chromatography matrices in the practice of the present

35   invention include synthetic polymers, e.g., polystyrene,
poly(methacrylates), etc.; cellulose, dextrans, agarose,
cross-linked agarose, and th like.   A presently

-24-

temperature in the range f about 15 up to 30°C will be employed, with temperatures in the range of about 20 up to 25°C preferred. Typically, elution time will vary as a function of column dimensions, matrix material, and the

5 like. Flow of eluent through the column will typically fall in the range of about 10 up to 300 cm/h.

Optionally, a portion of the eluate of the first hydrophobic interaction chromatography matrix which is eluted employing buffer having an elevated pH may be

10 applied to the same hydrophobic interaction chromatography matrix (after regeneration), and eluted a second time, employing the same two-stage or three-stage elution protocol described above. In this manner, additional quantities of the intact, monomeric,

15 correctly-folded IGF-1 are obtained. Those eluate fractions which contain substantial quantities of intact, monomeric, correctly-folded IGF-1, plus substantial quantities of other forms of IGF-1 (e.g., 20% or greater content of multimeric form(s) of IGF-1) are

20 likely candidates for such optional re-use of the first hydrophobic interaction chromatography matrix.

Next, the intact, monomeric, correctly-folded IGF-1-containing fractions of the eluates of the first hydrophobic interaction chromatography matrix may be then

25 contacted with a sufficient quantity of a second cation exchange matrix under conditions suitable to adsorb in the range of about 95 up to 100% of the IGF-1 peptides from the eluate. This is conveniently accomplished by passing the medium containing the IGF-1 peptides through

30 a column containing the second cation exchange material. This second cation exchange chromatography matrix is employed, where necessary, to remove "nicked" IGF-1 from IGF-1-containing fractions which contain "nicked" IGF-1, in addition to authentic IGF-1 and multimeric IGF-1. In

35 particularly preferred embodiments of the present invention, protease-deficient strains of methylotrophic yeast may b used t produce recombinant IGF-1, wherein

hydrophobic interaction chromatography matrix by
contacting the matrix first under conditions suitable to
remove some of the aberrant IGF-1 peptides from the
matrix, without removing significant quantities of the
5   intact, monomeric, correctly-folded form of adsorbed IGF-
1 from the matrix; and thereafter the matrix is contacted
under conditions suitable to remove substantially all of
the remaining adsorbed IGF-1 from the matrix. The IGF-1
content of eluate fractions can be determined by a
10   variety of techniques, e.g., HPLC.

The initial elution of some of the aberrant
IGF-1 peptides is accomplished by contacting the matrix
with in the range of about 1 up to 10 volumes, relative
to the volume of matrix, of a buffer system having a low
15   conductivity, i.e., a buffer system of less than about
100 mM salt. Exemplary buffer systems include acetate
buffers, phosphate buffers, lactate buffers, succinate
buffers, Bis-Tris buffers, and the like, as well as
mixtures thereto, having a pH of about 4.5. A quantity
20   of such buffer is employed so as to produce a
substantially salt-free eluate. Preferably, this initial
elution of the first HIC matrix involves the use of a
linear ammonium sulfate gradient starting at 20%
saturated ammonium sulfate buffered at pH 5.0 with 50 mM
25   sodium acetate/phosphate, and ending with 0% ammonium
sulfate buffered at pH 4.0 with the same buffer. This
may be followed by further washing using a pH 4.0 buffer
without ammonium sulfate.

Elution of the remaining adsorbed IGF-1 from
30   the matrix is accomplished by contacting the matrix with
in the range of about 1 up to 10 volumes of a buffer
system having an elevated pH, wherein said buffer is
employed in a quantity sufficient to raise the pH of the
eluate up to about 6.5-7.5.

35   Elution of IGF-1 from the IGF-1-containing
hydrophobic interaction chromotography matrix can be
carried out under a variety of conditions. Typically, a

-26-

prior to application to the second cation exchange
matrix.

Prior to use, the second cation exchange matrix
can be activated, or after use, the second cation
5 exchange matrix can be regenerated, employing the
following sequential wash procedure:

in the range of 1-10 column volumes of water,

in the range of 3-10 column volumes of a 0.5 M
sodium hydroxide solution,

10 in the range of 1-10 column volumes of water

in the range of 3-10 column volumes of a 50%
aqueous methanol solution, and finally

in the range of 1-10 column volumes of water;
and thereafter, the column is equilibrated with

15 in the range of 3-5 column volumes of 0.5 $\underline{M}$
sodium acetate, pH 4.5, and

in the range of 10-20 column volumes of 0.05 $\underline{M}$
sodium acetate, pH 4.5.

Once substantially all of the IGF-1 has been
20 adsorbed onto the second cation exchange matrix, and
prior to elution of the IGF-1 therefrom, it is desirable
to contact the IGF-1-containing matrix with in the range
of about 1 up to 5 volumes, relative to the volume of
matrix, of a dilute buffer of a weak acid. Weak acids
25 contemplated for this purpose include acetic acid and
phosphoric acid. A presently preferred dilute buffer of
a weak acid comprises a 0.05 $\underline{M}$ sodium acetate solution
having a pH of about 4.5. This optional wash serves to
remove impurities which are not as tightly bound to the
30 second cation exchange matrix as is the IGF-1.
Preferably, this optional wash step involves use of a
0.05 M sodium acetate solution having a pH of about 4.5
followed by a 0.05 M sodium acetate solution having a pH
of about 5.5.

35 Once substantially all of the IGF-1 has been
adsorbed onto the second cation exchange matrix, and
optionally washed as described above, IGF-1 is then

the levels of "nicked" IGF-1 may be practically

n gligible, in which case this second round of cation

exchange chromatography may be optional, and, following

hydrophobic interaction chromatography, the purification

5   method may proceed to a gel filtration chromatography

step, as described herein, if it is necessary to separate

authentic IGF-1 from multimeric IGF-1.

Cation exchange matrices contemplated for use

in this contacting step of the present invention are

10 strong cation exchange matrices capable of high

resolution, i.e., capable of resolving nicked IGF-1 from

intact, monomeric IGF-1. Exemplary cation exchange

matrices include carboxymethylated and sulfonated cation

exchange media. A presently preferred cation exchange

15 matrix for use in this contacting step of the present

invention is a sulfonated agarose (e.g., Fast-Flow

S-Sepharose or Toyopearl SP550C).

Quantities of the second cation exchange matrix

employed in the practice of the present invention can

20 vary widely. Typically, in the range of about 0.05 up to

1 liter of matrix per gram of IGF-1 in the medium being

treated will be employed.

Contacting of the IGF-1-containing broth with

the second cation exchange matrix can be carried out

25 under a variety of conditions. Typically, such

contacting is carried out for a time of at least about

0.1 minute and a temperature in the range of about 2 up

30°C; with temperatures in the range of about 20 up to

25°C being preferred.

30   Optionally, to reduce conductivity, if

necessary, the medium applied to the second cation

exchange matrix can be diluted with at least one volume

of water (or a low conductivity buffer such as the column

equilibration buffer) prior to contacting with the second

35 cation exchange matrix. The pH of the diluted IGF-1-

containing medium is preferably adjusted to about 4.5

-28-

eluate from either the first hydrophobic interaction
chromatography matrix or the second cation exchange
chromatography matrix that contained, as the predominant
form of IGF-1, intact, monomeric, correctly folded IGF-1,
5   is contacted with a sufficient quantity of a gel
filtration medium under conditions suitable to resolve
substantially all of the monomeric IGF-1 from the
multimeric forms. This is accomplished by passing the
medium containing IGF-1 through a column containing the
10 gel filtration medium. Gel filtration media contemplated
for use in the practice of the present invention include
size exclusion media having suitable pore size so as to
allow differentiation between the desired intact,
monomeric, correctly-folded form of IGF-1, and multimeric
15 forms thereof. Exemplary gel filtration media include
Sephadex, Sephacryl, Superdex, polymer-based resins, and
the like.

       Quantities of the gel filtration medium
employed in the practice of the present invention
20 typically are in the range of 1-10 l of gel filtration
medium per gram of IGF-1 in the media being treated.

       Contacting of the IGF-1-containing media with
the gel filtration medium can be carried out under a
variety of conditions. Typically, such contacting is
25 carried out for a time of at least about 120 minutes and
a temperature in the range of about 20-25°C.

       After use, the gel filtration medium can be
regenerated employing the following sequential wash
procedure:
30   In the range of 0.5-2.0 column volumes of
water, in the range of 0.5-2.0 column volumes of 0.5 M
NaOH, in the range of 0.5-2.0 column volumes of 50%
methanol, and finally, in the range of 0.5-2.0 column
volumes of water; and thereafter, the column is
35 equilibrated within the range of 1-5 column volumes of
0.05 M ammonium acetate, pH 6, or 0.2 M acetic acid.

eluted from the IGF-1-containing matrix by contacting the
matrix with a sufficient quantity of a buffer system
having a sufficient ionic strength so as to
differentially displace substantially all of the IGF-1
5   peptides from the matrix. Typically at least one volume
of eluent is employed for this purpose, with in the range
of about 3 up to 12 volumes being preferred.

A convenient way to accomplish this
differential displacement is to employ a sodium chloride
10   gradient in a buffered solvent system. For example, a
sodium acetate buffer having a pH of 5.5 can be employed
with increasing amounts of sodium chloride added to the
buffer over time. Thus, a linear gradient can be
provided starting with a first solvent system comprising
15   a 0.05 $\underline{M}$ sodium acetate solution (pH 5.5) with increasing
quantities of a second solvent system added thereto,
wherein the second solvent system comprises a 0.05 $\underline{M}$
sodium acetate/0.3 $\underline{M}$ sodium chloride solution
(pH 5.5).

20   Elution of IGF-1 from the IGF-1-containing
cation exchange matrix can be carried out under a variety
of conditions. Typically, a temperature in the range of
about 2 up to 30°C will be employed with temperatures in
the range of about 20 up to 25°C being preferred.
25   Typically, elution times will vary as a function of
column dimensions, matrix material, and the like. Flow
of eluent through the column will typically fall in the
range of about 10 up to 300 cm/h.

The eluate of the second cation exchange matrix
30   can be treated either by a second hydrophobic interaction
chromatography step, or, preferably, by a gel filtration
process. Such treatment is employed to remove residual
amounts of multimers from the substantially purified IGF-
1-containing medium.

35   When gel filtration is employed to remove
residual amounts of multimers from the substantially
purified IGF-1-containing medium, the portion of the

folded IGF-1 is optionally dilut d with a sufficient
volume of a buffered salt-containing solution having a pH
of about 4.0 to about 7.0, preferably about pH 4.5-5.0 so
as to render the salt concentration of the diluted eluate
in the range of about 0.4 up to 1.0, preferably 0.6 M.

The portion of the eluate of the second cation
exchange matrix which contains substantial quantities of
intact, monomeric correctly-folded IGF-1 is then
contacted with a second hydrophobic interaction
chromatography matrix, which can be the same as or
different than the first hydrophobic interaction
chromatography matrix previously used.

Quantities of the second hydrophobic
interaction chromatography matrix and conditions of
contacting for this step are similar to those employed in
the previous contacting of the IGF-1-containing medium
with the first hydrophobic interaction chromatography
matrix.

Once substantially all forms of IGF-1 (i.e.,
those forms which are still present at this stage of the
purification procedure) have been adsorbed by the second
hydrophobic interaction chromatography matrix, the IGF-1
is eluted from the matrix by again contacting the matrix,
as previously described for the first hydrophobic
interaction chromatography matrix. Thus, the matrix is
treated first, under conditions suitable to remove
substantially all remaining forms of IGF-1 other than the
intact, monomeric, correctly-folded form of IGF-1 from
the matrix, without displacing significant quantities of
the intact, monomeric, correctly-folded form of IGF-1;
and thereafter, the matrix is treated under conditions
suitable to remove substantially all of the remaining
adsorbed IGF-1 from the matrix.

Elution of forms of IGF-1 other than the
intact, monomeric, correctly-folded form of IGF-1
(predominantly multimeric forms of IGF-1) is accomplished
employing a buffer system having a low conductivity.

Once the IGF-1-containing medium has been
applied to the gel filtrati n medium, IGF-1 is then
eluted from the gel filtration medium by contacting the
gel filtration medium with a sufficient quantity of

5   eluent so as to facilitate differential movement of
multimeric and monomeric IGF-1 through the gel filtration
medium without significant, irreversible adsorption of
protein to the matrix. Typically, at least 1 volume of
eluent is employed for this purpose, with in the range of

10   about 1 up to 1.5 volumes being preferred. Exemplary
eluents for use in eluting IGF-1 from the gel filtration
medium include salt-containing buffers such as 50 mM
ammonium acetate, pH 6.0, or low conductivity buffers
such as 0.2 M acetic acid. While essentially any gel

15   filtration material having a suitable pore size may be
chromatographed with a salt-containing buffer as
described herein, it is especially preferred to conduct
gel filtration chromatography using a gel filtration
matrix which employs a polymer-based resin, most

20   preferably Toyopearl HW50F (TosoHaas, Philadelphia, PA),
and to elute with an acetic acid solution, such as 0.2 M
acetic acid.

Elution of IGF-1 from the IGF-1-containing gel
filtration medium can be carried out using various

25   conditions. Typically a temperature in the range of
about 20-25°C will be employed. Typically, elution times
will vary as a function of column dimensions, gel
filtration medium and the like. Flow of eluent through
the column will typically fall in the range of 5 up to 50

30   cm/hr.

When a second hydrophobic interaction
chromatography matrix is employed instead of gel
filtration, and prior to contacting the portion of the
eluate of the second cation exchange matrix with a second

35   hydrophobic interaction chromatography matrix, the eluate
of the second cation exchange matrix which contains
substantial quantities of intact, monomeric correctly-

-32-

diafiltration, and the like, while concentration of the
medium containing purified product can be accomplished by
lyophilization, diafiltration, and the like.

5   The medium from which IGF-1 is recovered
according to the invention method for IGF-1 purification
can vary widely. The recovery of natural, synthetic
and/or recombinant materials is presently contemplated.
Preferably, medium containing at least about 0.01 grams
of IGF-1 peptides per liter of medium will be employed
10   for the practice of the present invention.

           Synthetic sources of IGF-1 from which intact
IGF-1 monomer can be recovered and purified in accordance
with the present invention include recombinant modified
yeast, bacteria and/or mammalian cells containing one or
15   more DNA sequences operably encoding IGF-1 peptides.
Presently preferred are yeast species selected from the
genus _Pichia_, wherein the yeast are transformed with at
least one DNA fragment capable of expressing IGF-1;
especially where IGF-1 is expressed from a construct
20   comprising, in the direction of transcription, the
following DNA sequences:

            (i)   a promoter region of a methanol responsive
                  gene of _P. pastoris_, e.g., the _AOX1_
                  promoter,

25   (ii) a DNA sequence encoding a polypeptide
                  consisting of:

                  (a) the S. cerevisiae alpha mating factor
                       (AMF) pre-pro sequence, including a
                       processing site selected from the
30   group consisting of lys-arg; and lys-
                       arg-(glu-ala)$_x$, wherein x is an
                       integer between 1 and about 3, and

                  (b) an insulin-like growth factor-1
                       (IGF-1) peptide; and

35   (iii)   a transcription terminat r functional
                        in _P. pastoris_,

-31-

Such a buffer will prom te the elution of primarily
multimeric forms of IGF-1. An exemplary buffer system
useful for such purpose is a linear salt gradient of a
buffered solution having a pH of about 4.5 in a quantity
5   sufficient to produce a substantially salt-free eluate.
Preferably, a linear ammonium sulfate gradient starting
at 20% saturated ammonium sulfate buffered at pH 5.0 with
0.05 M sodium acetate/phosphate and ending with 0%
ammonium sulfate buffered at pH 4.0 with the same buffer
10 is used for this purpose. This may be followed with
further washing using pH 4.0 buffer without ammonium
sulfate.

        Elution of the remaining adsorbed IGF-1 from
the matrix is accomplished employing a buffer system
15 having a pH higher than the pH of the aqueous medium used
to equilibrate the HIC matrix. A convenient means to
provide such a medium is a linear gradient of a
substantially salt-free buffered solution having an
initial pH of about 4.0, wherein the pH of this buffer
20 solution is gradually raised to about 6.5-7.5.
Alternatively, the medium may be a buffered solution
having a pH of about 6.5 to about 7.5.

        As previously described with reference to the
first hydrophobic interaction chromatography matrix, a
25 portion of the eluate of the second HIC matrix which is
eluted employing elevated pH buffer, and containing
predominantly multimeric forms of IGF-1, plus small
amounts of intact, monomeric, correctly-folded IGF-1, can
be reapplied to the second hydrophobic interaction
30 chromatography matrix, then re-eluted employing the same
elution protocol described above.

        The substantially purified product obtained
from the above-described multi-step process can
optionally be treated to remove residual salts from the
35 purifi d product, and to concentrate the medium
containing the purified product. For xample, salt
removal can be accomplished by gel filtration,

-34-

employed for this purpose sh uld have a low conductivity, and should be of about the same pH as the media used to equilibrate the first cation exchange matrix.

The invention will now be described in greater

5 detail with reference to the following non-limiting examples.

EXAMPLES

EXAMPLE 1: CHARACTERIZATION OF RECOMBINANT PROTEINS

10   IN THE BROTH OF IGF-1-SECRETING STRAINS

OF PICHIA PASTORIS

Recombinant IGF-1 was produced by growing IGF-1-secreting strains of P. pastoris, G+IMB204S14 and M+IMB206S1 (described in U.S. patent application Serial

15 No. 578,728, filed September 4, 1990, and U.S. Serial No. 678,916, filed April 1, 1991, and International Application No. PCT/US91/06452, filed September 4, 1991), in a 10-liter fermentation conducted according to the following three-stage high cell density batch

20 fermentation procedure:

1) growth on excess glycerol,

2) growth on limited glycerol, and

3) growth on limited methanol.

Cells are initially grown on glycerol in a

25 batch mode. Because glycerol strongly represses the AOX1 promoter, the IGF-1 gene, which is regulated by this promoter, is not expressed during this phase. Following exhaustion of the glycerol, a limited glycerol feed is initiated. Glycerol does not accumulate during this

30 phase, but cell mass increases, and the AOX1 promoter is de-repressed. Finally, in the third phase, a methanol feed is initiated which fully induces the AOX1 promoter for the production of IGF-1.

Correctly folded, intact monomeric IGF-1 was

35 purified from the broth of 10-liter fermentations using the invention combination of cation exchange

wherein said DNA sequences are operationally associated
with one another for transcription of the sequences
encoding said polypeptide.

The specific _Pichia pastoris_ strains
5   G+IGF201S1, G+IGF201S2, G+IGF201S6, G+IGF201S10,
G+IGF202S3, G+IGF202S5, G+IGF204S2, G+IGF204S8,
G+IGF206S2, G+IGF206S5, G+IGF206S8, G+IGF206S9,
G+IMB202S2, G+IMB204S14, G+IMB206S1, G+IMB206S3,
G-IMB206S1, G-IMB206S2, or G-IMB206S3 are presently
10 preferred because they have proven to produce high levels
of IGF-1 upon fermentation. These specific presently
most preferred strains are prepared and caused to express
IGF-1 as described in copending application Serial No.
578,728, to which application the reader is directed for
15 additional detail as to the preparation of the strains
and expression of IGF-1 therefrom. Strains of P.
pastoris which are deficient in proteolytic activity and
which produce high levels of IGF-1 and reduced levels of
nicked IGF-1 upon fermentation are described in co-owned
20 United States Application Serial No. 678,916, filed April
1, 1991 and co-owned PCT International Application No.
US91/06452, filed September 4, 1991, the disclosures of
which applications are incorporated herein by reference.
An especially preferred P. _pastoris_ strain, which
25 produces high levels of authentic IGF-1 and reduced
levels of nicked IGF-1, is M+IMB206S1.

When the IGF-1 to be purified is contained in
the fermentation broth from a fermentation operation, it
is preferred to separate cellular and particulate
30 material from the fermentation broth prior to the initial
contacting of the IGF-1-containing medium with the first
cation exchange matrix. Preferably, the IGF-1 to be
purified is contained in a substantially cell-free
fermentation broth from a high cell density fermentation
35 operation. In this situation, the broth is optionally
dilut d with a buffered medium prior to contacting the
broth with the first cation exchange matrix. The buffer

adjusted to 2.7-2.8. After 4 hours, the pH decreased to
the set point value as a result of cellular metabolism.
The glycerol feed was then terminated and a methanol feed
(100% methanol plus 12 ml/L PTM₁) was initiated at 20

5   ml/hour. At this point in the fermentation of strain
M+IMB206S1, the set point of the pH controller was
adjusted to 2.8-3.0. After 4 hours of methanol feeding,
the methanol feed rate was increased to 60 ml/hour and
maintained at this rate for a total of approximately 72

10 hours, at which point the vessel was harvested.

B.   Characterization of Recombinant Proteins
in Fermentation Broth

Pichia-produced IGF-1 exists as several forms,

15 including intact, monomeric, correctly-folded
IGF-1, in the broth of fermentations of IGF-1-expressing
P. pastoris strains, as evidenced by immunoblot and HPLC
analyses of cell-free broth. Since the HPLC analysis
(employing the protocol described in Example 3A1) of

20 crude cell-free broth from the fermentation of
P. pastoris strain G+IMB204S14 and M+IMB206S1 does not
adequately resolve the various IGF-1 species, in order to
distinguish the IGF-1 species by HPLC, the native
P. pastoris proteins were removed through pretreating the

25 broth by small-scale cation exchange chromatography.

1.   Pretreatment of crude
fermentation broth

Direct injection of crude P. pastoris broth

30 onto HPLC usually did not result in a chromatogram with
distinct peaks. In order to directly analyze the
components of crude broth by HPLC (employing the protocol
described in Example 3), a clean-up procedure was
developed for removing endogenous P. pastoris

35 contaminants using a small-scale cation exchange
chromatography step. Several cation exchange systems
were tested for this purpose: sulfylpropyl cation

chromatography, hydrophobic interaction chromatography (HIC) and gel filtration chromatography.

A. Ten-Liter Fermentations of P. pastoris Strain G+IMB204S14 and M+IMB206S1

5   A 15-liter fermentor (Biolafitte; Princeton, NJ) containing 3.5 liters of 10x basal salts (42 ml 85% phosphoric acid/l, 1.8 g calcium sulfate·$2H_2O$/l, 28.6 g potassium sulfate/l, 23.4 g magnesium sulfate·$7H_2O$/l,

10   6.5 g potassium hydroxide/l) and 220 g glycerol in a total volume of 5.5 liters was sterilized. After the fermentor cooled, 24 ml PTM_1 trace salts (6.0 g cupric sulfate·$5H_2O$/l, 0.08 g sodium iodide/l, 3.0 g manganese sulfate·H_2O/l, 0.2 g sodium molybdate·$2H_2O$/l, 0.02 g boric

15   acid/l, 0.5 g cobalt chloride/l, 20.0 g zinc chloride/l, 65.0 g ferrous sulfate·$7H_2O$/l, 0.20 g biotin/l, 5.0 ml sulfuric acid/l) were added and the pH was adjusted to 5.0 with the addition of 28% (concentrated) ammonium hydroxide. The pH was controlled with the addition of

20   the same solution. Foaming was controlled with the addition of a 5% solution of Struktol J673. Temperature was maintained at 30°C, and dissolved oxygen was maintained above 20% of saturation by increasing agitation, aeration, reactor pressure or by

25   supplementation of the air feed with oxygen. Inocula were prepared from cells of P. pastoris strain G+IMB204S14 or M+IMB206S1 grown overnight in buffered yeast nitrogen base (YNB) (11.5 g/L KH_2PO4, 2.66 g/L K_2HPO_4, 6.7 g/L yeast nitrogen base, pH 6) containing 2%

30   glycerol. The fermentor was inoculated with 500-700 mls of the cultured cells which had grown to an OD_{600} of 2-8, and the batch growth regime was continued for 18-24 hours. At the point of glycerol exhaustion, indicated by an increase in dissolved oxygen concentration, a glycerol

35   feed (50% w/v glycerol plus 12 ml/L PTM_1) was initiated at 100 ml/hour. At this point in the fermentation of strain G+IMB204S14 the set point of the pH controller was

-38-

broth were carefully added to the column in an effort to
avoid disruption of the column bed. Although some
suspension of the absorbent usually occurred during
addition of liquid to the column, it was not detrimental
5   to sample binding or eluting. Broth samples and buffer
were allowed to flow through the column by gravity. The
IGF-1 was eluted with 2 ml of a 0.05 M sodium acetate, pH
5.5, buffer containing 1 M NaCl. The first milliliter of
eluate contained 80-90% of the total IGF-1, eluted by the
10 2 ml of elution buffer. Periodically (approximately
every 5-10 samples), the column was regenerated after the
salt elution with a 50% methanol wash. Less often, the
column was also regenerated with 0.5 M NaOH. These
columns retain their selective binding properties through
15 many successive uses.

2.   **Characterization**

The eluate obtained after small-scale cation
exchange chromatography of cell-free broth from a
20 fermentation of strain G+IMB204S14 or M+IMB206S1 contains
all of the IGF-1 species (i.e., nicked, misfolded,
multimeric and intact, correctly-folded monomer), which
can be resolved by the HPLC protocol described in Example
3. The different peaks detected in the chromatogram from
25 HPLC analysis of broth that had been subjected to cation
exchange chromatography correspond to different forms of
IGF-1 produced in recombinant P. pastoris fermentations.
The identity of the proteins corresponding to these peaks
was established by HPLC, immunoblot and SDS-PAGE analyses
30 of broth and broth components (isolated by HPLC, gel
filtration, cation exchange and hydrophobic interaction
chromatography).

A chromatogram from HPLC analysis (conducted as
described in Example 3A1) of broth from a fermentation of
35 an IGF-producing P. pastoris strain contains a peak
corresponding t protein that eluted from the HPLC column
at approximately 10 minutes which represents correctly

-37-

exchange capsules (FMC (Pinebr ok, NJ) and Cuno (Meriden, CT)) and the use of a bulk cation exchanger (e.g., Sepharose Fast Flow (Pharmacia, Uppsala, Sweden), SP-Spherodex (IBF, Columbia, MD), or Toyopearl SP650M and

5　SP550C (TosoHaas, Philadelphia, PA)) in a 2-ml disposable polypropylene column (0.8 x 2.4 cm, BioRad, Richmond, CA) with an integrated 10-ml reservoir. Any of these systems yielded satisfactory results. The two systems routinely used to pretreat crude broth for quantitative HPLC

10　analysis of the IGF-1 levels employed the Cuno cation exchange capsule or the SP-Spherodex or SP550C cation exchangers in a column format. The crude broth was cleaned up by cation exchange chromatography and injected directly onto an HPLC column. The resulting chromatogram

15　clearly resolved the different IGF-1 species in broth.

　　　　a.　**Pretreatment using cation exchange capsules**
　　　　　　The Cuno capsule is a 25-mm disk. The disk was first washed with 4 ml of 0.2 $\underline{M}$ acetic acid, then

20　equilibrated with 4 ml of 0.02 $\underline{M}$ acetic acid. A volume of crude cell-free broth (1-10 ml) was diluted 1:2 with 0.02 M acetic acid and loaded onto the disk. After loading, the disk was washed with 1.5 ml of 0.02 $\underline{M}$ acetic acid and the IGF-1 was eluted with 4 ml 0.02 M sodium

25　acetate, pH 5.5, plus 1 M NaCl. The first 1.5 ml of eluate contained 75-80% of the total IGF-1 and was usually the only elution volume collected. The capsule could then be regenerated by washing with 4 ml of 100% methanol.

30
　　　　b.　**Pretreatment using bulk cation exchanger in a column format**

　　　　　　To the disposable column, 0.25 ml of
35　prehydrated cation exchanger was added. The absorbent was first washed with 2 ml of 0.2 M acetic acid, then equilibrated with 2 ml of 0.02 M acetic acid. A volume of broth (1 ml) was loaded onto the column which was then washed with 1 ml of 0.02 M acetic acid. All buffers and

-40-

protein sequencing of this protein also confirmed that
the molecule was intact since only one amino-terminal
sequence, that of authentic IGF-1, could be identified.
Furthermore, reduced samples of this putative misfolded
5 form of IGF-1 which had been treated with dithiothreitol,
co-elute with reduced samples of authentic IGF-1 when
subjected to reverse phase HPLC. These results suggest
that this form is a misfolded species of IGF-1.

The proteins that elute from an HPLC column at
10 approximately 10.5-11.5 minutes were identified as nicked
or degraded forms of IGF-1 (i.e., IGF-1 molecules
containing two or more peptide fragments, generated by
cleavage of one or more peptide bonds, and held together
by disulfide bonds). There appears to be at least two
15 peaks by HPLC chromatographic analysis of cleaned up
broth that correspond to nicked forms of IGF-1. The
protein represented by the major peak (eluting at 10.7
minutes) was isolated during the S-Sepharose cation
exchange step of the IGF-1 purification process (see
20 Example 2C).

In silver-stained SDS-PAGE gels of non-reduced
samples of this isolated IGF-1 species, the molecule co-
migrated with IGF-1 standard and appeared as a single
band. However, in silver-stained gels of reduced samples
25 of the isolated material, a doublet was detected
representing two peptides of approximately 3-4 kDa each
(approximately half the size of intact IGF-1). The
position of this doublet in the gel corresponded to that
of the lower of the two bands detected below the band
30 representing intact IGF-1 in gels containing reduced
samples of crude cell-free broth. These results indicate
that this molecule is cleaved or nicked and held together
by disulfide bonds. Amino-terminal protein sequence
analysis of the protein confirmed that the molecule was
35 nicked prior to residue 40 since two amino-termini were
detected: one beginning with residue 1 f IGF-1 and one
beginning with residue 40 of IGF-1. Immunoblot analysis

folded, intact IGF-1 monomer. The identity of this
protein was confirmed initially on the basis of its
elution time by HPLC, which is identical to that of
standard recombinant IGF-1 (Amgen, Thousand Oaks, CA).
5   Furthermore, the protein with an HPLC elution time of
approximately 10 minutes was purified, as described in
Example 2 and subjected to additional analyses. SDS-PAGE
analysis of reduced and non-reduced samples of the
purified protein yielded identical results, revealing
10 that it is a 7.7-kDa intact protein that co-migrated with
IGF-1 standard. Immunoblot analysis of the purified
protein demonstrated that it is reactive with an antibody
generated against the last 14 amino acids of IGF-1. Gel
filtration chromatography of the purified protein
15 revealed that it elutes as expected for an IGF-1 monomer
of the correct size. Finally, amino acid analysis
confirmed that the amino acid ratios of the purified
protein correspond to those of standard IGF-1. Protein
sequence analysis showed that the complete amino acid
20 sequence of the purified protein is identical to that of
authentic IGF-1.

         The protein that elutes from an HPLC column at
approximately 8.6 minutes was tentatively identified as
misfolded IGF-1. This protein was isolated by HPLC and
25 hydrophobic interaction chromatography, and characterized
by SDS-PAGE, immunoblot and protein sequence analysis
(see Examples 3C and 3F for protocols). SDS-PAGE
analysis of reduced and non-reduced samples of this
protein showed that this form migrated with authentic
30 monomeric IGF-1 and that it was not a nicked form of IGF-
1 (i.e., that it was an intact protein in which the
primary structure was held together solely by peptide
bonds, as opposed to a cleaved IGF-1 molecule consisting
of two or more peptide fragments held together by
35 disulfide bonds). Western blot analysis of this protein
using an antibody directed against the C-terminus of IGF-
1 showed that it was immunoreactive. Amino-terminal

EXAMPLE 2:   **PURIFICATION OF CORRECTLY FOLDED, INTACT IGF-1 MONOMER FROM P. PASTORIS FERMENTATION BROTH**

The process for the purification of correctly
5   folded, intact monomeric IGF-1 from P. pastoris
fermentation broth was based on a combination of cation
exchange, hydrophobic interaction and gel filtration
chromatography. This process was applied to the
purification of correctly-folded, intact, monomeric IGF-1
10   from the broth of 10-liter fermentations of strain
G+IMB204S14 and M+IMB206S1. The material obtained after
each step in the purification of IGF-1 was qualitatively
and quantitatively (by HPLC; see Example 3A for a
description of the protocol) analyzed to determine the
15   IGF-1 yield and purity of the product. The results of
these analyses are tabulated in Tables I and II.

-41-

of reduced and non-reduced samples of this isolated
nicked IGF-1 molecule revealed that it is less reactive
than intact IGF-1 with the antibody directed against the
C-terminus of IGF-1.

5   Two additional nicked species were identified
in protein sequence analysis of IGF-1 recovered from the
first cation exchange chromatography step of the
purification process (see Example 2A). Either or both of
these species could correspond to the minor peak in the
10   HPLC chromatogram of cleaned-up cell-free broth (protein
eluting at 11 minutes). The amino-terminus of the
C-terminal fragment of one of these nicked molecules
begins at residue 25 of IGF-1. The amino terminus of the
C-terminal fragment of the other nicked species detected
15   in the broth of strain G+IMB204S14 begins at residue 14
of IGF-1.

          The last set of proteins detected by HPLC of
cell-free broth, which elute from the HPLC column after
11.5-16 minutes, appears to be disulfide-bonded multimer
20   forms of IGF-1. The presence of disulfide-bonded IGF-1
multimers in P. pastoris broth was indicated in SDS-PAGE
gels of broth and immunoblots of the gels. The putative
multimers migrated as IGF-1 dimers and trimers on non-
reduced SDS-PAGE gels and were reactive with antibody
25   directed against the C-terminus of IGF-1 in immunoblots.
When these multimers were reduced, they co-migrated with
standard monomer IGF-1 in SDS-PAGE gels and co-eluted
with monomer IGF-1 in HPLC, which indicates that they
contain disulfide-bonded IGF-1 monomers. Furthermore,
30   multimer IGF-1 (apparent dimer and trimer) species were
isolated on a gel filtration column and analyzed by HPLC
(see Example 3B). The isolated multimer eluted from the
column at 12-14 minutes, which corresponds to the elution
times of the proteins in broth that were proposed to be
35   multimers of IGF-1.

-44-

A.   Recovery of IGF-1 fr m Fermentation Br th

Pichia-produced IGF-1 was recovered from crude
fermentation broth using either a CUNO radial flow Zeta
Prep SP-250 (sulfylpropyl) cation exchange capsule or a

5   5-cm column packed with Toyopearl SP550C ion exchanger
(TosoHaas, Philadelphia, PA).

1.   Preparation of cell-free broth

Approximately 10 liters of a fermentor culture

10   of P. pastoris strain G+IMB204S14 or M+IMB206S1 was
centrifuged at 6500 x g for 20 minutes to separate broth
from the cells. Approximately 5 to 6 liters of cell-free
supernatant from broth of G+IMB204S14 was obtained
containing 400-600 mg of intact IGF-1 monomer, as

15   measured by HPLC (see Example 3A). An additional 40-60
mg of intact IGF-1 monomer was recovered from the cell
pellet by washing the pellet with approximately 4-5
liters of 20 mM acetic acid and again removing the cells
by centrifugation. The supernatant from the first

20   centrifugation was diluted 1:1 with 20 mM acetic acid and
combined with the cell pellet wash. Approximately 7.3 l
of cell-free broth was recovered from crude broth of
M+IMB206S1 and contained approximately 976 mg of
authentic IGF-1. The complete cell- free broth

25   preparations were then filtered through a Whatman GF
(glass fiber) filter.

2.   Preparation of cation exchanger
a.   Cation exchange capsule

30   A radial flow ZetaPrep SP-250 (sulfylpropyl)
cation exchange capsule (CUNO, Meriden, CT) was
equilibrated by pumping through the capsule three column
volumes (750 ml) of 0.2 M acetic acid, followed by four
column volumes of 20 mM acetic acid at a flow rate of 50

35   ml/minute. All steps in this first cation exchange
contacting were conducted at approximat ly 4°C.

-43-

TABLE I

RESULTS OF PURIFICATION OF IGF-1 FROM CELL-FREE
FERMENTATION BROTH OF P. PASTORIS STRAIN G+IMB204S14

Purification Step	Intact IGF-1 Monomer by HPLC			Volume
	mg	Percent purity	Percent Recovery	liters
Cell-Free Broth	525	ND	100	6.0
Recovery from SP-250 Capsule	425	34	81	0.84
Butyl HIC	235	74	45	0.59
S-Sepharose	203	91	39	0.20
Final HIC	147	>97%	28	0.32

ND = not determined

TABLE II

Purification of IGF-1 from Cell-Free Broth of
P. pastoris Strain M+IMB206S1

STEP	PROTEIN grams (BCA)	TOTAL IGF-1 grams (HPLC)	AUTHENTIC IGF-1 grams (HPLC)	OVERALL YIELD (%)
CELL-FREE BROTH	81.0	2.443	0.976	100
SP-RECOVERY	5.0	1.607	0.729	75
BUTYL HIC	1.006	0.673	0.613	63
S-SEPHAROSE	0.860	0.580	0.531	54
GEL FILTRATION	0.620	0.490	0.490	49
CONCENTRATION/ DIAFILTRATION	0.420[a]	0.463	0.453	46

[a] The final sample was analyzed by amino acid analysis.

-46-

b. **Toyopearl SP550C cation exchange chromatography column**

The cell-free broth from fermentation of M+IMB206S1 was applied to the SP550C column at a flow rate of 50-75 ml/minute. The column was then washed with 1 l of 20 mM acetic acid at 50 ml/min followed by two 1-1 washes with 50 mM sodium acetate, pH 5.0, and 50 mM sodium acetate, pH 5.5, respectively. Alternatively, the column was washed with four separate 1-1 washes all at a flow rate of 50 ml/min: 20 mM acetic acid, 50 mM sodium acetate, pH 5.5, 0.05 M NaCl in 50 mM sodium acetate, pH 5.5, 0.1 M NaCl in 50 mM sodium acetate, pH 5.5.

4. **Elution of IGF-1**
   a. **Cation exchange capsule**

The total IGF-1 was eluted by washing the capsule with six column volumes of a buffer comprising 20 mM sodium acetate, pH 5.5, and 1 M NaCl (at a flow rate of 50 ml/minute). Fractions equivalent to one column volume (250 ml) were collected during the washes employing 0.2 and 1.0 M NaCl-containing buffers, and assayed by HPLC to identify the IGF-1-containing fractions.

b. **Toyopearl SP550C cation exchange chromatography column**

IGF-1 was eluted from the column with a 2-liter linear gradient solution consisting of 0 to 0.5 M NaCl in 50 mM sodium acetate, pH 5.5. Eluate fractions (25-30 ml each) were collected during the wash and elution of the column. Alternatively, IGF-1 was eluted with a solution consisting of 0.3 M NaCl in 50 mM sodium acetate, pH 5.5.

5. **Characterization of recovery**
   a. **Cation exchange capsule**

The first fractions eluting during the 0.2 M NaCl wash of the SP-250 ZetaPrep capsule that had been

-45-

b.   Toyopearl SP550C cation exchange
chr matography column

Alternatively, a 5-cm diameter column was
packed with 250 ml Toyopearl SP550C ion exchanger in 0.5

5   M NaCl at a rate of 100 ml/minute (300 cm/hr). After
packing, a bed height of 12 cm was measured for a bed
volume of 235 ml. The column was regenerated with six
separate washes: 1 l of 1 M NaCl followed by 250 ml of
water, both at a flow rate of 50-75 ml/min, 1 liter of

10 0.5 M NaOH at 10-20 ml/min, 250 ml water at 25-50 ml/min,
1 l of 50% methanol at 10-20 ml/min, 250 ml water at 25-
50 ml/minute. For storage, the column was placed in 20%
ethanol at room temperature. To prepare the column for
loading, 500 ml of 200 mM acetic acid was applied to the

15 column at 50-75 ml/min followed by equilibration with
1.5-2.0 l of 20 mM acetic acid at 50-75 ml/min. All
operations on this column were carried out at room
temperature.

3.   Sample application and wash
a.   Cation exchange capsule

20

The diluted, filtered cell-free broth from a
fermentation of G+IMB204S14 (approximately 17 liters) was
pumped onto the ZetaPrep capsule at a flow-rate of
50 ml/minute. Analysis of the flow-through obtained

25 during loading of the cell-free broth onto the cation
exchange capsule revealed that most of the colored
material and native P. pastoris proteins in the broth
passed through the capsule, as determined by SDS-PAGE and
visual observation, whereas most of the total IGF-1

30 (> 95%) in the broth was retained by the capsule, as
determined by HPLC analysis of the flow-through.

After the diluted broth was loaded onto the
capsule, the capsule was washed at a flow rate of 50
ml/minute with two column volumes of a buffered solution

35 comprising 20 mM acetic acid followed by four column
volumes of 20 mM sodium acetate, pH 5 (20 mM acetate plus
10 M NaOH), containing 0.2 M NaCl.

-48-

purification: (1) a level of less than 10% nicked IGF-1
in the fraction, and (2) a concentration greater than 100
mg/l for the authentic IGF-1 in the fraction. Based on
these criteria and HPLC analyses of the remaining eluate
5   fractions, fractions 120-155 were pooled, and the pool
was prepared for further purification in the next step of
the process. HPLC analysis of this pool revealed that
authentic IGF-1 was the predominant IGF-1 species
contained in the pool. Approximately 730 mg of the
10   initial 976 mg of authentic IGF-1 contained in the broth
was recovered in a volume of 1 liter for a yield of 75%
for this step. This pool contained 21% misfolded IGF-1
(347 mg), 30% multimeric IGF-1 (488 mg), as well as 45%
authentic IGF-1 (730 mg). Although not resolved in the
15   chromatogram of this pool, nicked IGF-1 was also present
in the pool at a level of approximately 40-50 mg (2-3%).
At least 300 mg of nicked IGF-1 were removed from the
preparation at this step as well as 400 mg of multimeric
IGF-1.
20   A comparison of the total protein levels of the
recovered pool (fractions 120-155), as determined by BCA
(Pierce, Rockford, IL) assays, and the starting broth
revealed that 94% of the total protein that was present
in the cell-free broth had been removed from the
25   preparation during the initial recovery step.

         B.   **First Hydrophobic Interaction**
              Chromatography Step
              Following recovery of IGF-1 from cell-free
30   P. pastoris broth using cation exchange chromatography,
the IGF-1 was further purified by hydrophobic interaction
chromatography (HIC) using TSK Butyl Toyopearl-650M
matrix (TosoHaas, Philadelphia, PA). All steps in this
first HIC contacting were conducted at 20-25°C.

35

         1.   **Column preparation**

loaded with cell-fr e broth contained very little intact
monomer IGF-1 but a significant amount of nicked and
multimer IGF-1. Intact monomer IGF-1 started to elute in
the final fraction of the 0.2 M NaCl wash and the first
5   three to four fractions from the 1 M NaCl wash. The
eluate fractions from the 1 M NaCl elution were pooled.
The pool contained approximately 425 mg of partially
purified intact monomeric IGF-1 in a volume of 840 ml.
Therefore, in the initial cation exchange step of the
10 IGF-1 purification process conducted to recover IGF-1
from crude broth, a significant amount of the undesired
nicked and multimer IGF-1 forms was eliminated from the
IGF-1 preparation. Approximately 81% of the intact
monomer IGF-1 was recovered in this step.

15

        b.   **Toyopearl SP550C cation exchange**
             chromatography column
        Immunoblot and HPLC analysis of the flow-
through obtained while loading the broth onto the column
20 revealed negligible amounts of authentic IGF-1 but
significant amounts of native Pichia protein contaminants
and multimeric IGF-1. Therefore, a large portion of
contaminant Pichia proteins did not bind to the matrix
and were removed from the broth by passing through the
25 column into the flow-through. In contrast, the majority
of the IGF-1 bound to the matrix.
        Quantitative HPLC analysis of eluate fractions
revealed that a significant amount of nicked IGF-1 was
removed from the preparation during the pH 5.5 wash
30 (fractions 41-80) and the first portion of the salt
gradient elution (fractions 81-119). As shown by HPLC
analysis of a pool containing fractions 101-119 collected
during the middle of the salt gradient elution, authentic
IGF-1 began to elute from the column in these fractions
35 which also contained misfolded and multimeric IGF-1.
        The following criteria were used in the
selection of the fractions that were pooled for further

a. Sample of IGF-1 being purifi d fr m broth of G+IMB204S14

Approximately 425 mg of partially purified intact, monomeric, correctly-folded IGF-1 obtained from

5   cell-free fermentation broth of strain G+IMB204S14 that had been passed through a Zeta-Prep cation exchange capsule was diluted to a total volume of three liters by addition of a buffer containing 5 $\underline{M}$ sodium chloride, 0.5 $\underline{M}$ sodium acetate, and 0.5 M sodium phosphate, pH 4.0.

10   One liter of an 80% saturated solution of ammonium sulfate was then slowly added to the three liters of diluted material. This resulted in a turbid solution containing precipitated IGF-1. Precipitate was removed by centrifuging the solution for 20 minutes at 10,000 rpm

15   in a GSA rotor. The precipitate was dissolved in one liter of an acetate/phosphate buffer at pH 4.5. The four liters of soluble material (supernatant obtained after centrifugation) was adjusted to pH 4.5 and combined with the resuspended precipitate to yield a total of five

20   liters. This 5-liter solution contained IGF-1 (425 mg), ammonium sulfate (16% of saturation), 40 mM sodium acetate, 40 mM sodium phosphate, pH 4.5 and 0.4 $\underline{M}$ NaCl, and was loaded onto the HIC matrix. All solutions were applied to the column containing the HIC matrix at a flow

25   rate of about 80-300 cm/hr, as per manufacturer's recommendations. To avoid precipitation of IGF-1 in subsequent steps of the purification process, the pH of the IGF-1-containing material was maintained at a pH of 4.5 or greater.

30

b. Sample of IGF-1 being purified from broth of M+IMB206S1

Eluate fractions from the initial SP550C cation exchange chromatography step that contained authentic

35   IGF-1 at a specified level of purity (fractions 120-155) were pooled. This pool, which contained approximately 730 mg of partially purified authentic IGF-1 in a volume

-49-

A variety of different conditions were investigated for the binding of IGF-1 t the HIC matrix, with particular attention paid to the concentration of ammonium sulfate in the medium and the pH of the medium.

5   Typical of conditions used for HIC, IGF-1 was found to bind to the HIC matrix at high ammonium sulfate concentrations (i.e., greater than about 15% of saturation or about 0.6 $\underline{M}$ $(NH_4)_2$ SO_4). Therefore, equilibration of the HIC matrix included the addition of

10   ammonium sulfate.

A 2.5-cm diameter column was packed at 5-24 ml/min with approximately 37 ml or 123 ml of TosoHaas Butyl Toyopearl-650M matrix in water. The column packed with 37 ml of matrix had a bed height of 7.5 cm, whereas

15   the column packed with 123 ml of matrix had a bed height of 25 cm. The column was regenerated by washing with four or five separate washes at a flow rate of 800-1000 cm/hr: 200-250 ml of water, 200-800 ml of 0.5 N NaOH, 100-800 ml of 50% methanol, and 100-250 ml of water.

20   When the column was regenerated with five washes, a water (250 ml) wash was included between the NaOH and methanol washes. The matrix was then equilibrated with 200-750 ml of buffer (15-20% saturated ammonium sulfate, 50 mM sodium acetate, 50 mM sodium phosphate, pH 4.0-5.0). (One

25   liter of 100% saturated ammonium sulfate is defined as containing 533 grams of the solid salt.)   This ammonium sulfate concentration of the buffer is presently preferred for optimal binding of IGF-1 to the matrix. As is true for all buffered solutions described for use in

30   the purification of IGF-1 by HIC, the HIC binding buffer was prepared from a 10x concentrated solution of 0.5 M acetic acid and 0.5 M monobasic sodium phosphate titered with sodium hydroxide to the appropriate pH value.

35   2.   **Sample preparation and applicati n**

-52-

50 mM sodium phosphate at a flow rate of 80-300 cm/hr.
It is presently preferred that the ammonium sulfate
gradient solution is a linear gradient starting at 20%
saturated ammonium sulfate buffered at pH 5.0 with 50 mM

5   acetate/phosphate and ending without ammonium sulfate
buffered at pH 4.0 with the same buffer. This is
followed by 400 ml of pH 4.0 buffer without ammonium
sulfate. Because increasing the pH of the column
surprisingly decreased the hydrophobic interactions in

10   this system, in the next elution step, the pH was
increased from about 4.0-4.5 to about 6.5-7.5 using a
0.5-2 liter buffer gradient (50 mM sodium acetate, 50 mM
sodium phosphate) containing no ammonium sulfate.

15   4.   **Characterization of recovery**

            a.   **Recovery from first HIC step**
                 in purifying IGF-1 from broth
                 of strain G+IMB204814

            Eluate fractions (approximately 7.5 ml) were
20   collected during each step of the elution process and
analyzed by HPLC to identify IGF-1-containing fractions.
The data were compiled and the elution profiles for each
form of IGF-1 are shown graphically in Figure 1. All of
the misfolded IGF-1 eluted from the HIC matrix during

25   elution of the column with the ammonium sulfate gradient
solution (fractions 0-60). Approximately 75% of the
multimer was removed from the matrix during the ammonium
sulfate gradient elution (fractions 0-60) and during the
first part of the pH gradient elution (fractions 60-84).

30   However, the correctly-folded intact and nicked monomeric
forms of IGF-1 remained bound to the matrix. During the
second part of the elution step using a pH gradient
solution (fractions 85-125), the intact monomer and
nicked forms of IGF-1 eluted from the column.

35   The fractions separated into groups 1 and 2 in
Figure 1 were pooled into pools 1 and 2, respectively,
for further purification. The first pool c nsisted of

of 1 liter, was diluted to 2 liters by addition of 200 ml
10X stock of sodium acetate/ph sphate pH 5.0 buffer and
800 ml of water containing 160 g ammonium sulfate to
adjust the concentration of ammonium sulfate in the
5   sample to 15% of saturation. The pH was adjusted to pH
5.0 with NaOH or HCl before slowly adding the ammonium
sulfate solution. The IGF-1-containing solution was then
loaded onto the column at 15 ml/minute (180 cm/hr).

10   3.   **Elution of IGF-1**
          During the course of this work aimed at the
development of a method for the purification of IGF-1, we
surprisingly discovered that the pH of the elution buffer
employed in this HIC purification step altered the IGF-1
15   elution profile quite drastically. At pH 7.5, intact
IGF-1 monomer starts to elute in a salt elution gradient
at 0.6 $\underline{M}$ ammonium sulfate, and is completely eluted when
the ammonium sulfate concentration of the gradient is
decreased to 0.2 $\underline{M}$. At lower pH values, intact IGF-1 did
20   not begin to elute until the ammonium sulfate
concentrations of the gradient had been decreased to
lower values. If the pH of the buffer was less than 5,
preferably 4.5, then the ammonium sulfate concentration
could be reduced to 0% with very little intact IGF-1
25   monomer eluting from the column, although misfolded and
multimeric IGF-1 are removed from the matrix under such
conditions. Thus, decreasing the ammonium sulfate
concentration of the column at low pH resulted in
separation of misfolded and multimeric IGF-1 from
30   authentic IGF-1. Then, the authentic IGF-1 was eluted
from the matrix by increasing the pH.
          Based on these findings, proteins are typically
eluted from the HIC column in two or three steps. First,
the ammonium sulfate concentration of the column is
35   decreased from 20% of saturation to no ammonium sulfate
using a 500-ml ammonium sulfate gradient soluti n
buffered at pH 4.5-5.0 containing 50 mM sodium acetate,

-53-

fractions collected during the period beginning when the
ammonium sulfate concentration of the column was 0%
(fraction 60) and extending to the time at which the
majority of the multimer was removed from the matrix
5   (fraction 84). The second pool consisted of fractions 85
to 125 collected during the pH gradient elution step.
The chromatograms from HPLC analysis of these two pools
indicate that very little multimer and practically no
misfolded IGF-1 were present in the second pool, which
10   consisted primarily of IGF-1 monomer and nicked IGF-1.
Although not evident in HPLC analyses of diluted pool 2
samples, there was some residual multimer present in the
second pool, as determined by SDS-PAGE and immunoblot
analyses of these samples (see Example 3C).

15

b.   **Recovery from first HIC step in purifying**
IGF-1 from broth of strain M+IMB20681

Fractions (25-30 ml) were collected during each
step of the elution process and analyzed by HPLC (20-μl
20   injections) and SDS-PAGE and immunoblot to identify IGF-
1-containing fractions which could be pooled for further
processing. Misfolded IGF-1 was removed in the ammonium
sulfate gradient in fractions 1 through 18. The misfolded
IGF-1 eluted from the column at approximately 90% purity.
25   The stained gel of a non-reduced sample of the
pool of fractions 1-20 showed a broad band representing a
high molecular weight protein, a band corresponding to a
protein that comigrated with authentic IGF-1 monomer, and
a faint band between the other two bands that could be a
30   dimer form of IGF-1.
The pH 4 wash (containing no ammonium sulfate)
removed the majority of multimeric IGF-1 that was bound
to the resin. This 50 mM sodium acetate/phosphate pH 4
wash was applied to the column during the collection of
35   fractions 19 through 30. After the individual fractions
were characterized, fractions 21 through 29 were pooled
and characterized by analytical HPLC. In the resulting

chromatogram, the peak corresp nding to multimer IGF-1
represented 75% of the peak area in the chromatogram.
The chromatogram also revealed the presence of a small
amount of authentic (48 mg) and nicked (13 mg) IGF-1 in

5   this pool. The amount of authentic IGF-1 lost in this
step is most likely a function of the capacity of the
column.

When samples of the pH 4 wash pool (fractions
21-29) were examined by stained gel SDS-PAGE and western

10   blot analyses, the predominance of multimer forms was
evident. When not reduced, there were many high
molecular weight forms in the sample that were not
detected in reduced samples of the pool. Reduced samples
of the pool consisted primarily of material that co-

15   migrated with monomer IGF-1.

The criteria used in the selection of authentic
IGF-1-containing eluate fractions for further
purification in the next step of the protocol were as
follows: first, a level of less than 60% multimeric IGF-

20   1 present in the fraction and second, a concentration of
greater than 100 mg/L for the authentic IGF-1. Fractions
30-80, which were collected during the pH gradient
elution, were selected and pooled to apply in the next
step of the purification process. A total of 613 mg of

25   the 730 mg of authentic IGF-1 loaded onto the HIC column
was recovered in this pool. This yield corresponds to a
recovery of 84% for this process step and a cumulative
recovery of 63% from cell-free broth of strain
M+IMB206S1. HPLC analysis of this pool also showed a

30   level of 3 mg (0.5%) misfolded and 27 mg (4%) multimeric
IGF-1. Although nicked IGF-1 was not resolved in the
chromatogram, it was estimated that 21 mg (3%) of nicked
IGF-1 was present in the pool. The purity of the
authentic IGF-1 in this pool was therefore estimated at

35   92%. The overall level of protein in the recovered
eluat pool from the HIC step was measured at 1 gram

-55-

(Table II) or 20% of the total protein that was loaded onto the column.

Because all of the fractions collected during the pH gradient elution were pooled for further

5   purification, the gradient elution can be replaced with a single 50 mM sodium acetate/phosphate, pH 7.5 step elution. A pH step elution decreases the volume of the recovered IGF-1 to approximately one liter and greatly simplifies the elution process. Therefore, the majority

10   of the authentic IGF-1 is in the eluate collected during the pH step elution until the authentic IGF-1 concentration of the eluate is less than 100 mg/l. This pool can be combined with the final fractions of the pH 4 wash that contain authentic IGF-1.

15

5.   **Reapplication of sample to column and**
      characterization of recovery

Only approximately 40% (170 mg) of the SP-250 capsule-purified intact, correctly-folded monomer IGF-1

20   from broth of strain G+IMB204S14 that was loaded onto the HIC column was recovered in the second pool of eluate fractions from the HIC column. Figure 1 shows that a large amount of intact IGF-1 monomer (87 mg) was present in the first pool along with nicked and multimeric IGF-1.

25   To recover additional intact monomer IGF-1, the first pool of eluate fractions was loaded back onto the HIC column and eluted according to the same procedure used in the initial HIC purification. An additional 65 mg of intact monomer IGF-1 was recovered from the HIC column.

30   This pool 2 material was combined with the first pool 2 material to yield a final pool containing 235 mg of intact, correctly-folded monomer IGF-1 in a volume of 590 ml, at a purity of 74% (Table I). Therefore, approximately 55% of the SP-250 capsule-purified intact

35   monomer IGF-1 that was loaded onto the butyl HIC column was recovered, reducing the total recovery to 45% at this stage in the purification process. This HIC step of the

purification procedure thus effectively removed most of
the multimeric and misfolded forms of IGF-1 from the
preparation.

5   C.   Cation Exchange Chromatography
                The nicked and intact monomer IGF-1 species in
the material obtained from HIC of Pichia-produced IGF-1
were separated by cation exchange chromatography using
Fast Flow S-Sepharose (Pharmacia, Piscataway, NJ) matrix.
10 This matrix was prepared for chromatography by
regeneration in a column using the same protocol as that
described for regeneration of the HIC matrix (Example
2B). All steps in this second cation exchange
chromatography procedure were conducted at 20-25°C.

15

                1.   Preparation of column
                A 2.5-cm diameter column was packed at 2-20
ml/min with approximately 35-80 ml of Pharmacia Fast Flow
S-Sepharose in water. The column was equilibrated by
20 passing 100-250 ml of 0.5 M sodium acetate, pH 4.5,
followed by 300-750 ml of 50 mM sodium acetate, pH 4.5,
through the column. This low salt solution, buffered at
a pH low enough to provide a net positive charge on the
protein, was used to facilitate binding of IGF-1 to the
25 negatively charged matrix.

                2.   Sample preparation and loading
                The pool of IGF-1-containing eluate fractions
obtained from HIC purification of partially purified IGF-
30 1 from cell-free broth of strain G+IMB204S14 had a
conductivity of 7 mmho and contained approximately 235 mg
of intact IGF-1 monomer. The low-salt conditions used to
elute the IGF-1 from the HIC column minimized
manipulations of the eluate prior to ion exchange
35 chromatography. The pH of the pool of IGF-1-containing
eluate fractions from HIC purification of the IGF-1
sample from broth of strain G+IMB204S14 or strain

-57-

M+IMB206S1 was adjusted to 4.5 and the volume of the pool
was adjusted to on liter by addition of 50 mM sodium
acetate, pH 4.5. The HIC-purified IGF-1 was loaded
directly onto the cation exchange matrix, Fast Flow S-
5   Sepharose, without further manipulation. After the
protein was loaded on the column at 2-15 ml/min, the
column was washed with approximately 100 ml of 50 mM
sodium acetate, pH 4.5. The column was then additionally
washed with 330 ml of 50 mM sodium acetate, pH 5.5, which
10   additional wash is preferred.

3.   **Elution of IGF-1**
The IGF-1 was eluted from the column using a
500- or 1000-ml 0 to 0.3 M sodium chloride gradient
15   buffered with 50 mM sodium acetate, pH 5.5. The higher
pH reduced the ionic interaction between the proteins and
the matrix, and the eluate fractions containing intact,
correctly-folded, monomeric IGF-1, as determined by HPLC,
were pooled. The matrix was stored in 20% ethanol.

20

4.   **Characterization of recovery**
a.   **Recovery from second cation exchange
chromatography in purifying IGF-1
from broth of strain G+IMB204814**
25   The elution profile for each of the IGF-1
species, which was generated from HPLC analysis of eluate
fractions (approximately 8 ml each) collected during the
gradient elution, is shown in Figure 2. This cation
exchange chromatography step completely resolved the
30   nicked and intact monomer forms that were present in the
preparation. Eluate fractions 28-50, obtained when the
NaCl concentration of the gradient wash solution in the
column ranged from .15 to .25 $\underline{M}$, were pooled. The
results of SDS-PAGE, immunoblot, HPLC and gel filtration
35   analysis (see Examples 3A, 3B, and 3C for protocols) of
the pool of eluate fractions all show the presence of
residual multimer (HPLC and gel filtration quantitation

-58-

indicated a multimer level of 9%). Nevertheless, 86%
(203 mg) of the intact, correctly-folded IGF-1 monomer
that was loaded onto the Fast Flow S-Sepharose column was
recovered in the eluate pool and the overall recovery at
5   this stage of the purification process was 39% (Table I).

b.   **Recovery from second cation exchange**
      step in purifying IGF-1 from broth of
      strain M+IMB20681

10   Fractions (25-30 ml) were collected throughout
the elution process and analyzed by HPLC to identify IGF-
1-containing fractions which could be pooled for the next
step in the purification process. In the pH 5.5 wash
(fractions 6-17), approximately 10 mg of nicked IGF-1
15   were removed from the preparation. The presence of lower
molecular weight peptides, which migrate more rapidly
than IGF-1 standard, in an SDS-PAGE gel and corresponding
immunoblot of a reduced sample of this pool that are not
present in the non-reduced sample of the pool is
20   indicative of the presence of nicked IGF-1. Nicked IGF-1
is the size of intact monomer in the non-reduced state
but dissociates into two peptides upon reduction, each
peptide being smaller than the intact molecule.
            Fractions 21-29 were collected during the first
25   portion of the salt gradient elution. As was the case in
the early stages of the elution of the SP550C column with
the linear salt gradient in the recovery step, there
appeared to be some authentic IGF-1 eluting along with
additional nicked IGF-1 in the first portion of the salt
30   gradient elution of the S-Sepharose column. SDS-PAGE
analysis of this pool showed forms of IGF-1 that migrated
more slowly than IGF-1 standard under both non-reduced
and reduced conditions. It is possible that these may be
glycosylated or otherwise modified forms of IGF-1 which
35   are present in very low levels.
            The criteria used to select eluate fractions to
pool for further purificati n were as follows: a level

-59-

of less than 5% nicked IGF-1 present in the fraction and
a concentration of greater than 150 mg/L for the
authentic IGF-1. Fractions 30-45, collected during the
second half of the salt gradient elution, were selected
5 on the basis of these criteria and pooled to apply in the
next step of the purification process. A total of 531 mg
of authentic IGF-1 was recovered from the 613 mg of
authentic IGF-1 that was loaded onto the column for a
recovery yield of 87% for this process and a cumulative
10 recovery of 54% from cell-free broth of strain
M+IMB206S1. HPLC analysis of this pool showed that it
also contained 3 mg (0.5%) misfolded, 3 mg nicked (0.5%),
and 43 mg (7.5%) multimeric IGF-1.

     The overall level of protein in the recovered
15 pool from the S-Sepharose cation exchange chromatography
step was measured at 0.86 grams (Table II). The pool was
also analyzed by SDS-PAGE and immunoblot. The small
amount of multimer IGF-1 contained in the pool was
visible in the non-reduced SDS-PAGE stained gel.

20

     D.   **Second Hydrophobic Interaction**
           Chromatography Step or Gel Filtration
           Chromatography Step

     To reduce the multimer level in the otherwise
25 pure intact monomer IGF-1 preparation, the pool of eluate
fractions from the Fast Flow S-Sepharose cation exchange
chromatography of IGF-1 partially purified from broth of
strain G+IMB204S14 was loaded onto a regenerated TSK
Butyl Toyopearl-650 M matrix (see Example 2B for a
30 description of matrix regeneration) for the second and
final HIC step in the purification of IGF-1, as described
in Example 2.D.1. below. All steps of this second HIC
process were carried out at 20-25°C.

     It is presently preferred to remove residual
35 multimer from the purified IGF-1 using gel filtration
chromatography. As described in Example 3B also,
multimeric and monomeric forms of IGF-1 are well resolved

-60-

by gel filtration chromatography. The pool of eluate
fractions from the Fast Flow S-Sepharose cation exchange
chromatography of IGF-1 partially purified from broth of
the strain M+IMB206S1 was loaded onto a gel filtration
5 chromatography column as described in Example 2.D.2.
below.

    1. Second HIC step to remove multimer from
      pooled eluate from the second cation
10   **exchange step in the purification of**
      IGF-1 from broth of strain G+IMB204S14

     a. Sample preparation
     Prior to loading the pool of eluate fractions
15 onto the HIC column, the pool was diluted to 500 ml by
adding 50 ml of a buffer solution containing 0.5 $\underline{M}$ sodium
acetate and 0.5 $\underline{M}$ sodium phosphate, pH 4.5. To adjust
the pH of the solution to 4.5, a sufficient quantity of 1
$\underline{N}$ HCl was added. Water was then added to increase the
20 total volume to 438 ml, and ammonium sulfate (63 ml of a
solution which is 80% of saturation) was added slowly to
yield 500 ml of a solution containing IGF-1 (203 mg),
ammonium sulfate (10% of saturation), 50 mM sodium
acetate, and 50 mM sodium phosphate, pH 4.5. No
25 precipitate formed upon addition of ammonium sulfate to
the eluate.

     b. IGF-1 elution and reloading
     The two-step elution procedure described in
30 Example 2B was used to elute the IGF-1 from the HIC
column. The eluate fractions were combined into two
pools and, as described for the first HIC step (see
Example 2B), pool 1 was loaded back onto the HIC column
and eluted to recover intact IGF-1 monomer contained in
35 the pool. The pool 2 eluates from the two elutions of
the HIC matrix were combined to yield a single pool of
purified IGF-1 monomer.

-61-

c.   Characterization of rec very

HPLC analysis of the final eluate pool revealed
that this preparation of intact IGF-1 monomer was greater
than 97% pure and contained 147 mg of intact IGF-1
5   monomer in a volume of 320 ml for a final recovery of 28%
of the intact IGF-1 monomer that was present in the cell-
free broth (Table I).

2.   Gel filtration chromatography of pooled
10   eluate from the second cation exchange
step in the purification of IGF-1 from
broth of strain M+IMB20681

a.   Column preparation

15   A Superdex 75 16/60 prepacked 60 cm x 1.6 cm
diameter gel filtration column (Pharmacia, Uppsala,
Sweden) was regenerated using 60 ml water at 1.4
ml/minute, 120 ml of 0.5 M NaOH at a flow rate of 0.5
ml/minute, 60 ml of water at 0.5 ml/minute, 120 ml of 50%
20   isopropyl alcohol at 0.5 ml/minute, and 60 ml of water at
0.5 ml/minute. The column was then equilibrated with at
least two column volumes (250-350 ml) of 50 mM ammonium
acetate after regeneration or storage. (The column was
stored in 20% ethanol.) All operations using this column
25   were carried out at room temperature.

Alternatively, a 2.5 cm diameter column was
packed with Toyopearl HW50F size exclusion media
(TosoHaas, Philadelphia, PA) in 50 mM ammonium acetate,
pH 6.0, at a flow rate of 6 ml/min (73 cm/hr) to a bed
30   height of 92 cm (450 ml bed volume). The column was
regenerated by washing the column with 150 ml water, 300
ml 0.5 M NaOH, 150 ml water, 300 ml 50% methanol,
followed by 150 ml water. The flow rate during
regeneration was reduced to 2 ml/min due to high back
35   pressure during the hydroxide wash. The column was
stored in 20% ethanol and column operation was carried
out at room temperature.

-62-

b. Sample preparation and application

As described in Example 2.C.4.b, fractions
obtained during salt gradient elution of the S-Sepharose
column that contained authentic IGF-1 at a specified
5   level of purity were pooled. The pool (containing
approximately 530 mg authentic IGF-1) was then
concentrated from a volume of 420 ml to 60 ml in a 400 ml
Amicon pressure cell using a YM2 membrane that retains
proteins with a molecular weight greater than 2000 Da
10   (i.e., a 2000 m.w. cut-off). The 60-ml concentrated pool
was divided into 20 aliquots, each containing
approximately 30 mg of authentic IGF-1. The aliquots
were applied to the column individually, requiring a
total of 20 chromatography runs for the batch. During
15   sample application for each of the 20 cycles, the flow
rate of the buffer (50 mM ammonium acetate, pH 6.0) was
0.7 ml/minute. Fifteen minutes after loading, the flow
rate was adjusted to 1.4 ml/minute. After the monomer
and multimer were eluted from the column, the next
20   aliquot was loaded on the column, and the cycle was
repeated. A larger column with a higher capacity would
have reduced the number of cycles required.

Alternatively, when the Toyopearl HW50F column
is used for gel filtration chromatography to remove
25   residual multimer from partially purified IGF-1, 0.2 M
acetic acid can be used as the elution buffer.
Interestingly, IGF-1 monomer eluted more slowly when the
acetic acid buffer was used then when the 50 mM ammonium
acetate buffer was used. It appears that there is some
30   interaction involving this matrix and monomeric IGF-1
which retards the elution of monomeric IGF-1 and enhances
the resolution of monomeric and multimeric IGF-1.

c. Elution of IGF-1

35   The column eluate was monitored with an on-line
UV-absorbance detector set at a wavelength of 280 nm.
When protein began to elute from the column, as indicated

-63-

by the UV absorbance detector, fractions (7 ml each) of
eluate were collected every 5 minutes until authentic
IGF-1 monomer began to elute from the column, as
indicated by a sharp increase in UV absorbance. At that
5   point, 0.5-minute (0.7-ml) fractions were continually
collected for approximately 3 minutes so that the
transition fractions (i.e., fractions collected at the
interface of multimer and monomer elution) could be
analyzed. At the end of the 3-minute period, 5-minute
10   fractions (7 ml each) were collected for the remainder of
the monomer elution. Once the monomer had completely
eluted, another aliquot containing 30 mg of IGF-1 was
loaded onto the column and the elution process was
repeated.

15

d.   Characterization of recovery

The fractions that were collected from the gel
filtration column for all 20 cycles were analyzed by
HPLC. The IGF-1 material that was first to elute from the
20   column was collected during the period beginning
immediately after 64 ml of buffer had eluted from the
column and ending immediately after 100 ml of eluate had
eluted from the column (i.e., material in the 65th
through 100th milliliter of eluate). HPLC analysis of
25   this material that eluted from the gel filtration column
as the first peak did not detect any protein (i.e., the
HPLC chromatogram did not contain any peaks). Therefore,
the concentration of this high molecular weight
presumably multimeric IGF-1 species was estimated by
30   comparing the absorbance of this material at 280 nm to
that of the later eluting dimer IGF-1, which was
quantifiable by HPLC analysis. SDS-PAGE analysis of this
eluate pool showed a very high molecular weight protein
located at the top of the gel under non-reducing
35   conditions. Under reducing conditions, the band
corresponding to this high molecular weight protein was
of greatly diminish d intensity and a band corresponding

-64-

to monomer IGF-1 was present in the gel. In the western
blots of these gels, a very faint band could be seen at
the top of the blot containing the non-reduced sample,
and the monomer band could be seen in the blot of the
5   reduced sample.

The second peak on the gel filtration on-line
detector chromatogram corresponded to material that
eluted in the 100th through 120th milliliter of eluate.
The majority of this material had an apparent molecular
10 weight consistent with that of a trimer of IGF-1 as shown
under non-reduced conditions on SDS-PAGE gels and
corresponding western blots. In addition, this material
contained a higher molecular weight species, as detected
in these analyses. Under reducing conditions, the only
15 band observed in the gel and immunoblot of this eluate
pool corresponded to protein that co-migrated with
standard IGF-1. The HPLC chromatogram for this pool
revealed that IGF-1 trimer had a relatively long
retention time (i.e., elution occurred from 14 to past 16
20 minutes).

The third peak on the gel filtration
chromatogram corresponded to material that eluted in the
125th through 135th milliliter of eluate. HPLC analysis
and SDS-PAGE and western analysis demonstrated that this
25 eluate pool consisted primarily of IGF-1 dimer with small
amounts of monomer and trimer IGF-1 as well. On an HPLC
column, this pool eluted between 11 and 14 minutes.

Authentic IGF-1 started to elute in the 126th
milliliter of eluate, which also contained a fairly high
30 level of dimer IGF-1. However, the first 2.1 ml (1.5
minutes) of elution after the monomer began to elute
contained only 0.2 mg of monomer. During the subsequent
2.1 ml of elution, 3 mg of monomer IGF-1 eluted,
containing approximately 10% dimer IGF-1. This pool was
35 recycled into the IGF-1 pool being loaded onto the column
to improve the recovery yield of this process. If this
pool was not recycled by reloading onto the column, then

10% of the total mon mer IGF-1 would have been lost (3 mg
of the 30 mg for each load). At that point, after a
total of 130 ml of eluate had eluted, the multimer IGF-1
concentration had decreased to less than 3% and the
remaining eluate was recovered until the end of monomer
elution which occurred at the 150th milliliter of eluate.
The monomer pools obtained after each of the 20 separate
gel filtrations of the 20 aliquots of eluate from the S-
Sepharose step were pooled to yield a total of 481 mg
authentic IGF-1 in a volume of 600 ml. The yield for
this step was 91% and the total yield for the
purification process up to this point was 49% (Table II).
HPLC analysis of the monomer pool indicated a purity of
98% and a multimer level of 0.9%. SDS-PAGE and western
blot analysis of the monomer pool, in which only monomer
IGF-1 was detected under both reducing and non-reducing
conditions, was consistent with the results of HPLC
analysis of the pool.

**E. Diafiltration and concentration of
purified IGF-1**
   **1. IGF-1 purified from broth of
strain G+IMB204B14**

The final purified IGF-1 product obtained as
pooled eluate (320 ml) from the second HIC step was
concentrated to a volume of about 20 ml by filtration
through a filter that retains proteins larger than 2000
Da (YM2 filter, Amicon, Darvers, MD) in a 400-ml Amicon
pressure cell. The buffer of the concentrated IGF-1
preparation was exchanged with 0.1 M acetic acid by
diluting the concentrated IGF-1 to 400 ml with 0.1 M
acetic acid and then concentrating the protein again by
filtration to a total volume of approximately 20 ml.
This process was repeated twice to accomplish a nearly
complete buffer exchange. Diafiltration was conducted at
a temperature of 2-10°C.

-66-

2. IGF-1 purified from br th of strain M+IMB20681

All of the eluate fractions from the gel filtration chromatography step containing monomer IGF-1
5   were pooled to yield a total volume of 600 ml containing 481 mg authentic IGF-1. This pool was then concentrated to a volume of 50 ml in an Amicon pressure cell using a YM2 membrane (2,000 m.w. cut off). In order to remove the ammonium salt, the concentrated, purified monomer
10 pool was diluted 10-fold with 0.1 M acetic acid and reconcentrated. This dilution and concentration step was repeated twice. A final volume of 45 ml was removed from the pressure cell and the filter was washed with two 0.1 M acetic acid washes totaling 5 ml. The first 45 ml was
15 passed through a Corning 0.22 micron filter, and then the 5 ml collected from the filter wash was also filtered through the same filter and combined with the 45 ml to bring the total volume of final purified authentic IGF-1 to 50 ml. The amount of concentrated IGF-1 (Table II)
20 was determined at 453 mg by HPLC for a yield of 94% for the concentration step and a total yield of 46% for the entire purification process.

To ensure that ammonia was being removed by this process, the level of ammonia in each filtrate
25 collected during the concentration and dilution series was determined using an ammonia combination electrode (Corning, Medford, MA). A standard curve was generated with known ammonia concentrations. The ammonia concentrations were calculated at 53 mM for the gel
30 filtration buffer and 42 mM for the first filtrate obtained from the initial concentration step. Subsequent filtrates from the series of 10-fold dilutions and concentrations were calculated at 4.6, 0.016, and 0.001 mM ammonia.

35

-67-

**EXAMPLE 3: CHARACTERIZATION OF PICHIA-PRODUCED IGF-1
PURIFIED FROM FERMENTATION BROTH**

The eluate pools of purified IGF-1 obtained
after the second HIC step of several repetitions of the
5   procedure described above were concentrated in 0.1 M
acetic acid by diafiltration, as described in Example 2E.
Typical samples of intact, monomeric, correctly-folded
IGF-1, purified as described above from broth of strain
G+IMB204S14 and strain M+IMB206S1 were characterized and
10   quantitated by HPLC, gel filtration chromatography, SDS-
PAGE, immunoblot, amino acid composition and amino acid
sequence analyses.

A.   **HPLC Analysis**

15

1. **Protocol**

A Waters (Medford, MA) 600 solvent delivery
system, Waters Model 481 Lambda Max variable wavelength
detector, Wisp 710B auto-injector and a Schimadzu
20   Crom-Pac (Cole Scientific, Moorepark, CA) integrator
constituted the HPLC system. A Vydac C4 column
(0.46 X 5 cm) with a guard column was used to resolve
components of the <u>Pichia pastoris</u>-produced IGF-1
preparations. Samples obtained during the purification
25   process and samples of the final purified preparation of
IGF-1 were loaded onto the column at a flow rate of
1ml/min and were eluted in a trifluoroacetic acid
(TFA)/acetonitrile gradient. The eluant was prepared by
using mobile phase A (0.1% TFA) to dilute mobile phase B
30   (95% acetonitrile, 5% water, 0.1 % TFA). A 1%/minute
gradient of 25% to 42% mobile phase B was passed through
the column during a period of 17 minutes at a flow rate
of 1 ml/minute to elute the material that had been loaded
onto the column. The column was then regenerated with
35   100% mobile phase B at a flow rate of 2 ml/minute for 4
minutes followed by 25% mobile phase B for 4 minutes at 2
ml/minute. The flow rate was then reduced to 1 ml/minute

-68-

and the column was equilibrated for 2 minutes bef re
reinjection with another sample to be analyzed. The
detector was set at 0.05 absorbance units full scale
(AUFS) and a wavelength of 215 nm was used for maximum

5   sensitivity.

In order to quantitate the levels of Pichia-
produced IGF-1 by HPLC, known amounts of standard IGF-1
(Amgen) (0.5-5.0 μg) were injected onto the HPLC column
and the area under the corresponding peaks in the

10   chromotograms was measured. A standard curve was
generated by plotting area versus μg of IGF-1 loaded onto
the HPLC column. A conversion factor for use in
converting the area under HPLC chromatogram peaks to
IGF-1 was calculated from the standard curve. When the

15   detector was set at 0.05 AUFS and wavelength of 215 nm,
the conversion factor varied from 350 units/μg to 405
units/μg of IGF-1 injected onto the column. Using this
information, it was possible to determine the
concentration of correctly folded, intact, monomeric

20   IGF-1 present in a broth or purification sample by
measuring the area under the corresponding peak on the
chromatogram from HPLC analysis of the sample. This
conversion factor was also used to estimate the
approximate concentration of other IGF-1 species as well.

25   However, the absolute concentrations of each of these
other species may vary depending on differences in their
specific conversion factors.

2.   Analysis of purified sample

30   Several dilutions of purified IGF-1 were
analyzed by HPLC. The equivalents of 5, 2, 1, 0.5, 0.2,
and 0.1 μl of the concentrated purified IGF-1 were
injected onto the C4 column. The area of the peak
corresponding to intact, correctly-folded, monomeric IGF-

35   1 in chromatograms from the analysis of 0.1- to 1-2μl
aliquots was directly proportional to the volume f the
sample. Using an IGF-1 standard obtained from Amgen, a

-69-

standard curve was generated and used to calculate a concentration of 8.2 ± 1.4 mg/ml for the IGF-1 concentration of the purified preparation from broth of strain G+IMB204S14 and of 9.0 ± 0.3 mg/ml for the IGF-1

5 concentration of the purified preparation from broth of strain M+IMB206S1.

Purity estimates of the IGF-1 preparation increased with decreasing volume of the purified material injected onto the HPLC column. The chromatogram of a 2-

10 μl aliquot of sample purified from broth of G+IMB204S14 indicated that the preparation was 97.3% pure intact, correctly-folded IGF-1 monomer, with three minor contaminants, represented by a front shoulder peak (possibly misfolded IGF), a back shoulder peak (nicked

15 IGF), and the multimer region peaks, comprising 0.9%, 0.4% and 1.4% of the preparation, respectively. Purity determination based on HPLC analysis of a 1-μl aliquot indicated that the preparation was 98.7% pure correctly-folded, intact IGF-1 monomer with no multimer present,

20 whereas HPLC analysis of a 0.2-μl aliquot indicated that the preparation was 99.4% pure correctly-folded, intact IGF monomer. Because the multimer elutes from the HPLC column as a broad heterogeneous peak, it is difficult to estimate its presence by HPLC. However, 2 μl of the

25 concentrated purified IGF-1 is equivalent to almost 15 μg of IGF-1. Therefore, it would be expected that low-level contaminants would be detected in HPLC analysis of a 2-μl aliquot of purified IGF-1.

The chromatogram of a 2-μl aliquot of sample

30 purified from broth of strain M+IMB206S1 indicated that the preparation was 97.3% pure authentic IGF-1 with 1.1% multimer, 0.73% nicked and 0.9% putative misfolded IGF-1. The estimated purity of the 0.1-0.5 μl aliquots was greater than 99%.

35

B. Gel Filtration Chromatography Analysis

-70-

1. Protoc 1

A Superdex 75 HR 10/30 gel filtration column
(Pharmacia, Uppsala, Sweden) with a bed volume of 24 ml
(10 X 300 mm) was used to characterize the final purified

5   IGF-1. The HPLC system described above (see Example 3A)
was modified for use with this column. The detector was
set at a wavelength of 280 nm and a sensitivity of 0.05
AUFS. IGF-1-containing samples obtained during
purification of IGF-1 from broth were loaded onto the

10 column and a buffer containing 0.1 to 0.3 M ammonium
acetate, pH 6, was used at a flow rate of 0.5 ml/minute
to separate the IGF-1 species by size.

2. IGF-1 Analyses

15

a.   IGF-1 purified from broth of strain G+IMB204S14

To estimate the actual level of IGF-1 multimer
present in the purified IGF-1 preparation from broth of

20 strain G+IMB204S14, 50 μl of the concentrated purified
IGF-1 (approximately 250 μg), diluted to a volume of 200
μl with 0.3 M ammonium acetate, pH 6, was analyzed by gel
filtration chromatography. Integration of the two peaks
corresponding to the two faster eluting contaminants

25 (multimer IGF-1) indicated that the first peak (material
eluting at 22.67 minutes which was presumably trimer) was
1.0% of the total area and the second peak (material
eluting at 24.75 minutes which was presumably dimer) was
2.6% of the total area. The overall percentage of

30 multimer in the purified preparation was calculated at
3.6%, and the percentage of monomer (third fastest
eluting species in the chromatogram) was calculated at
94.9%. The material corresponding to these three peaks
was collected and separately analyzed by HPLC (see

35 protocol described in Example 3A).

From the HPLC chromatogram of material
corresponding to the monomer peak in the gel filtration

-71-

chromatogram, it was calculated that more than 95% of the
IGF-1 monomer that was loaded onto the gel filtration
column was recovered in the eluate. Approximately 19 μg
of intact monomer IGF-1 from the gel filtration column
5   was loaded onto the C4 column, and no multimer was
detected in the HPLC chromatogram.

The multimer forms of IGF-1 that eluted from
the gel filtration column as the two fastest eluting
species were also analyzed by HPLC, but the multimer
10   concentration was too low for detection on HPLC.

In order to analyze some of the multimer
species that might be present in low amounts in the
purified IGF-1, 200 μl of material from the first pool of
eluate fractions from the first HIC step of the
15   purification (see Example 2D) was subjected to gel
filtration chromatography (as described above) since it
contained a relatively high level of multimer. The
material corresponding to the individual peaks was
collected and separately analyzed by HPLC. HPLC
20   chromatograms of the components of the first pool of
eluate fractions from the first HIC step eluted from the
gel filtration column at 22.7 minutes (trimer) and 24.8
minutes (dimer), respectively. Material that eluted at
approximately 35.5 minutes from the gel filtration column
25   was also analyzed by HPLC, but no peaks were detected in
the HPLC chromatogram. Presumably, this is a small IGF-1
peptide that either binds too tightly to the C4 column,
so that it was not removed in the elution gradient, or
does not bind to C4 at all.

30

b. IGF-1 purified from broth of strain
M+IMB20681

The chromatogram from gel filtration chromato-
graphy analysis of 450 μg of the final preparation
35   purified from broth of strain M+IMB206S1 showed that it
contained 99.2% monomer and 0.8% multimer IGF-1. The

-72-

chromatogram of 200 μg of this preparation indicated that
it consisted of 100% monomer.

C.   SDS-PAGE and Immunoblot Analysis
 5   1. Protocols
                   a. SDS-PAGE
                      i. Electrophoresis conditions
        The tricine sodium dodecyl sulfate
polyacrylamide gel electrophoresis (tricine SDS-PAGE)
10 system [Schagger, H. and von Jagow, G. (1987) Anal.
Biochem. 166:368], a system optimized for the separation
of proteins ranging from 5 to 20 kDa, was used for
electrophoretic analysis of IGF-1-containing samples. In
brief, electrophoresis was conducted in the Mini-PROTEAN
15 II (BioRad, Richmond, CA) vertical gel system.
Separating gels were 13% T, 3% C, and the stacking gels
were 4% T, 3% C. As defined by Schagger and von Jagow
(supra), T denotes the total percentage concentration of
both monomers (acrylamide and bisacrylamide), and C
20 denotes the percentage concentration of the cross-linker
relative to the total concentration T.
        Electrophoresis samples were prepared by
placing them in a boiling water bath for 2-3 minutes
after adding sample buffer components to the following
25 concentrations: 2% SDS, 12% glycerol, 50 mM Tris HCl, pH
6.8, 0.0025% Coomassie Brilliant Blue G, and 0.001%
pyronin Y. In addition, the disulfide bonds of some
samples were reduced by the inclusion of 100 mM
dithiothreitol (DTT) in the sample buffer. A constant
30 volume of sample buffer was added to each well of the
gels, including the "blank" lanes. Electrophoresis was
conducted at a maximum 100 volts until the tracking dyes
reached the bottom of the gels. The gels were then
processed for either Western blot analysis or for protein
35 staining.

-73-

ii. Fixing and C massie-staining tricine gels

Protein visualization by Coomassie staining was conducted by incubating the gels in 50% ethanol, 10%
5   acetic acid, 5% TCA and 200 mg/L Coomassie Brilliant Blue R-250 for 30 minutes. Due to the ethanol contained in this solution, the gels partially dehydrated after Coomassie staining. Therefore, following staining, the gels were rehydrated in 10% ethanol, 7% acetic acid, 1%
10 TCA and 50 mg/L Coomassie Brilliant Blue R-250. To covalently fix the proteins in the gels, the gels were incubated for 30 minutes in 10% glutaraldehyde. Following the final incubation, the gels were washed extensively with distilled water to remove the
15 glutaraldehyde. The washing procedure typically involved soaking the gels in distilled water for two hours, during which time fresh water was added at least twice, followed by an overnight incubation of the gels in several volumes of distilled water.

20   ## b. Immunoblot

Purified protein and cell-free broth protein samples that were analyzed by immunoblot were first separated by tricine gel electrophoresis (see Example 3.C.1.a) and then electroblotted onto 0.1 μm
25 nitrocellulose in a solution of Towbin buffer (25 mM Tris HCl, pH 8.3, 190 mM glycine, 20% methanol). Before electrotransfer, protein gels were equilibrated for 30 minutes in Towbin transfer buffer. Non-reduced gels were equilibrated in Towbin buffer containing 1%
30 β-mercaptoethanol.

After the proteins were transferred onto nitrocellulose, the filter was incubated for one hour in blocking buffer (0.25% gelatin, phosphate-buffered saline, 0.05% Tween 20, 0.02% sodium azide). Rabbit
35 anti-IGF-1 antisera 10A, generated against a C-terminal IGF-1-peptide, was diluted 1:2000 with bl cking buffer and incubated with the filter overnight. The filter was

washed with blocking buffer for an hour and incubated with ^{125}I-Pr tein A (0.02 μCi/ml) for 45 minutes. After one hour of washing with blocking buffer, the filter was air dried and exposed to X-ray film with an intensifying

5   screen at -75°C.

2. Analyses
a. SDS-PAGE
i. IGF-1 purified from broth of
10   strain G+IMB204814

Non-reduced and reduced samples of the purified IGF-1 containing 5, 2, 1, 0.5, 0.2, and 0.1 μg of IGF-1 as determined by amino acid composition analysis were subjected to electrophoresis, and Coomassie staining.

15   The minimum amount of reduced or non-reduced IGF-1 that could be detected in Coomassie-stained SDS-PAGE gels was 0.2 μg. A single band corresponding to a protein that co-migrated with standard IGF-1 was visible in gels containing non-reduced and reduced samples (0.2, 0.5, 1

20   or 2 μg) of purified IGF-1. However, two bands, one corresponding to a protein that co-migrated with standard IGF-1 and one corresponding to a protein that migrated more slowly than standard IGF-1, were visible in the gels of the non-reduced and reduced 5-μg samples of IGF-1.

25   The more slowly migrating protein in the non-reduced 5-μg sample was identified as a multimer of disulfide-bonded IGF-1 monomers. The amount of multimer present in the purified IGF-1 preparation was estimated at 4-10% of the total IGF-1 based on a visual comparison of the

30   intensities of the bands corresponding to IGF-1 multimer and known amounts of purified IGF-1.

The faint band detected above the band corresponding to monomeric IGF-1 in gels of reduced 5 μg samples of purified IGF-1 migrated to the same position

35   as does multimeric IGF-1. Additional SDS-PAGE analyses f reduced samples of purifi d IGF-1 hav been conducted under more stringent reducing conditions, and this band

-75-

was not detected. Furthermore, this band was pr sent on
the corresponding Western blot (see Example 3C2c) of
reduced samples of purified IGF-1, indicating that it is
a multimeric form of IGF-1. Therefore, this upper band

5   is most likely the result of incomplete reduction of the
IGF-1 prior to electrophoresis. Bands corresponding to
proteins migrating more rapidly than monomer IGF-1, which
would indicate the presence of nicked or degraded IGF-1,
were not detected in the gel of the reduced samples.

10 Because no other bands were seen on the reduced gel, the
level of purity for the purified IGF-1 is most likely
greater than 95%.

                    ii. IGF-1 purified from broth of
15   strain M+IMB206S1

            Aliquots of the preparation purified from broth
of strain M+IMB206S1 containing 200, 500, 1000, and 2000
ng authentic IGF-1 were loaded into lanes of an SDS-PAGE
gel.   The non-reduced samples showed a faint band

20 corresponding to multimer IGF-1 on the silver-stained
gel; however, this species was barely visible in western
blots of less than 2000 ng of the purified preparation.
The level of multimer could not be determined from a
silver-stained gel since the staining intensity was not

25 proportional to the level of protein, especially for low
level contaminants. Another band was seen just above the
monomer band in the lanes of the silver-stained gel
containing 1000 and 2000 ng of non-reduced IGF-1. This
species was present at a level less than that of the

30 multimer contaminant. The SDS-PAGE gel of the reduced
samples did not show any contaminants except for a very
light band at the multimer position in the lane
containing 2000 ng of IGF-1.

                    b. Scanning densitom try
35
            To more accurately determine the multimer
concentration from the results of SDS-PAGE analyses of

purified IGF-1, the gels f reduced and non-reduced
samples of IGF-1 purified from broth of strain
G+IMB204S14 were subjected to scanning densitometry using
a Hoefer (San Francisco, CA) scanning densitometer at a

5   fixed wavelength of 580 nm. Densitometer tracings were
generated by scanning the gels of non-reduced 5-µg and 2-
µg samples and reduced 5-µg and 2-µg samples. The two
peaks in the tracing generated by scanning the gels of
non-reduced samples correspond to multimer and monomer

10   IGF-1, respectively. The small peaks in the densitometer
tracings generated by scanning the gels of reduced
samples of purified IGF-1 correspond to incompletely
reduced IGF-1 multimer, whereas the large peaks in the
same tracings correspond to reduced multimer plus

15   monomeric IGF-1. The calculated areas for the monomer
peaks corresponding to 0.2 up to 5 µg of purified, non-
reduced IGF-1 were plotted against the amount of IGF-1 to
establish a standard curve. The curve was linear between
0.2 to 2 µg of IGF-1. However, the peak corresponding to

20   the multimer band in the gel containing 5 µg of non-
reduced IGF-1 could still be used to calculate the amount
of multimer present since the multimer in this lane
represented less than 2 µg. The area for the 5 µg
multimer peak was used to calculate an amount of 0.63 µg,

25   which is equivalent to a multimer concentration of 12.7%.
A multimer amount of 0.07 µg was calculated based on the
area of the peak corresponding to a multimer in 2 µg of
non-reduced IGF-1 which is equivalent to a multimer
concentration of 3.4%. Tracings generated by scanning

30   the gels of non-reduced samples of the purified
preparation containing less than 2 µg of IGF-1 indicated
that the material was 100% pure monomeric IGF-1. It
should be noted that there was a high amount of
background in the tracing generated by scanning the gels

35   of non-reduced purified IGF-1, and this background was
included in the area calculations f r the multimer peaks.
Thus, the values calculated for the multimer

-77-

concentration of purified IGF-1 based on scanning
densitometry are over-estimati ns of the actual
concentration.

c. Immunoblot analysis of IGF-1 purified from broth of strain G+IMB204814

5   Tricine SDS-PAGE gels of reduced and
non-reduced samples of purified IGF-1 (0.1, 0.2, 0.5, 1,
2 and 5 μg of intact IGF-1 monomer based on HPLC analyses
10   of purified IGF-1) were also subjected to immunoblotting.
Estimates of IGF-1 multimer and monomer concentration
based on immunoblot analyses are dependent on the
relative binding of multimer and monomer to the
polyclonal antisera used in the analyses, and evidence
15   exists for a greater binding of antisera by multimer as
compared to monomer IGF-1. Therefore, immunoblot
analysis of purified IGF-1 is a more sensitive method for
the detection of IGF-1 multimer than SDS-PAGE. However,
when estimating the amount of multimer in the purified
20   IGF-1 by immunoblot analysis, it is necessary to keep in
mind the possible effects of relative antibody binding.

         In the immunoblot of non-reduced samples of
purified IGF-1, bands corresponding to multimer IGF-1
were detected in lanes containing Amgen IGF-1 standard as
25   well as in the 5-, 2- and 1- μg samples of purified IGF-
1. The multimer band in the lane containing 2 μg of
purified IGF-1 appeared to be similar in intensity to the
monomer band in the lane containing 0.1 μg of purified
IGF-1 (which would equate to a multimer level of 5%) and
30   of much less intensity than the monomer band in the lane
containing 0.2 μg of purified IGF-1 (multimer level
<10%). Visual estimates of the IGF-1 multimer content of
the purified IGF-1 by western blot analysis of non-
reduced samples (5-10%) compare well with values obtained
35   by visual estimation of Coomassie-stained SDS-PAGE gels
of the non-reduced samples.

-78-

Similar to the Coomassie-stained SDS-PAGE gels of reduced sampl s of purified IGF-1, the immunoblot of the reduced samples also showed the incomplete reduction in the 5 μg sample. Most importantly, all the bands that

5   appeared in the Coomassie-stained SDS-PAGE gels of purified IGF-1 were present in the immunoblots of the samples. This indicates that there are no non-IGF-1 contaminants present in the purified preparation, and that the only readily measurable contaminant present in

10 the purified IGF-1 is multimeric IGF-1.

D. **Summary of HPLC, Gel Filtration chromatography, SDS-PAGE and Immunoblot Analyses of IGF-1 purified from broth of**

15 **strain G+IMB204B14**

The percentages of IGF-1 multimer in the purified IGF-1 preparation determined by HPLC (1.4%), gel filtration chromatography (3.6%), SDS-PAGE (4-10%), immunoblot (5-10%) and densitometry of Coomassie-stained

20 SDS-PAGE gels (3.4-12.7%) all seem to be in fairly close agreement considering the low levels of multimer present in the preparation. Because 250 μg of IGF-1 was loaded onto the gel filtration column, this technique was probably the most accurate for determining the multimer

25 levels of the purified IGF-1. Furthermore, there is the possibility that sample preparation for SDS-PAGE analysis caused aggregation of some of the monomeric IGF-1 in the purified preparation resulting in higher estimates of the multimer level. No such sample preparation was performed

30 for gel filtration chromatography. No non-IGF-1 protein contaminants were detected when the stained gels and immunoblots of samples of the purified IGF-1 were compared.

Although multimeric IGF-1 could be identified

35 by a variety f techniques used to measure purity of the IGF-1 preparation, th nicked and misfolded forms of IGF-1 were differentiated and detected only by HPLC. The

percentage of nicked IGF-1 was measured to be about 0.4%
by HPLC, and 0% by SDS-PAGE and immunoblot analysis of
reduced, purified IGF-1. HPLC analysis also showed a
contaminant appearing as a front shoulder of the peak

5 corresponding to intact, correctly-folded, monomeric IGF-
1. This contaminant was present at a level of 0.9%, and
is presumed to be misfolded form(s) of IGF-1.

E. Amino Acid Analysis

10

1. Protocol

Purified IGF-1 was acid hydrolyzed and the
resultant amino acids characterized on a Beckman (Palo
Alto, CA) 6300 Amino Acid Analyzer. To acid hydrolyze

15 IGF-1 protein, carefully measured volumes of purified
IGF-1 solution were added to 6 x 50 mm glass tubes and
dried in a Savant (Farmingdale, NY) Speed Vac. These
tubes were placed in a reaction flask containing 6 N HCl.
Oxidation was minimized by applying a vacuum and sealing

20 the flask. The flask was placed overnight in a 110°C
oven, and the protein was hydrolyzed by the hot HCl
vapors.

Following hydrolysis, the reaction flask was
cooled to room temperature, and the hydrolysis tubes were

25 removed. Any HCl that may have condensed in the tubes
was removed by drying in a Speed Vac. The free amino
acids were dissolved in a minimum of 100 μl Beckman Amino
Acid Sample Dilution Buffer, Na-S, for loading in the 50-
μl loop of the analyzer. A Nelson (Cupertino, CA) 3000

30 Series Chromatography Data System was used for
quantitation by comparing the integrated chromatograms of
amino acid standard solutions and the resuspended,
hydrolyzed samples.

35   #### 2. Analyses

a.   IGF-1 purified from br th f
strain G+IMB204814

A protein concentration of 5.15 mg/ml was
determined from eight separate analyses of purified IGF-1
5   using norleucine as an internal standard. A standard
deviation of 0.26 mg/ml was also calculated. Therefore,
186 to 206 mg of protein was contained in the total
volume of 38 ml of purified IGF-1. Table III shows the
estimated and actual amino acid ratios. The values
10   calculated for the numbers of cys, met, and tyr residues
were all lower than the actual values due to oxidative
destruction of these amino acids under conditions of
hydrolysis. The higher value calculated for the number
of threonine residues was most likely due to peak
15   integration errors resulting from incomplete resolution
of this residue and the adjacent serine residue. These
deviations are within the expected limits of this method.

-81-

TABLE III

AMINO ACID COMPOSITION ANALYSIS OF IGF-1 PURIFIED FROM BROTH OF STRAIN G+IMB204S14

Amino Acids	Published Compositions[a]	Injections								Estimated Average of Tests 1-8
		#1	#2	#3	#4	#5	#6	#7	#8	
Asp (+Asn)	5	5.5	5.1	5.1	5.1	5.2	5.0	5.1	5.1	5.1
Thr	3	5.2	5.2	4.8	5.4	4.0	4.0	4.1	4.2	4.6
Ser	5	4.6	4.1	4.6	4.1	4.2	4.2	4.3	4.4	4.3
Glu (+Gln)	6	6.9	6.6	6.7	6.6	6.1	6.5	6.5	6.5	6.5
Pro	5	5.0	5.7	0.0	6.1	0.0	5.7	6.0	6.0	4.3
Gly	7	7.5	6.8	6.5	6.8	6.7	6.7	6.8	6.7	6.8
Ala	6	6.4	6.4	6.8	6.5	6.5	6.4	6.4	6.4	6.5
Cys	6	.0	0.0	0.0	0.0	1.4	1.7	1.9	2.0	.9
Val	3	2.9	2.7	2.6	2.5	2.4	2.2	2.2	2.2	2.5
Met	1	.0	0.0	0.0	.1	.3	.1	.1	.1	.1
Ile	1	.4	.4	.3	.3	.4	.4	.4	.4	.4
Leu	6	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Tyr	3.	1.9	2.3	1.9	1.8	2.1	1.9	1.6	1.6	1.9
Phe	4	3.6	3.9	3.5	3.5	3.9	3.8	3.5	3.5	3.6
His	0	.2	0.0	.2	.2	.5	.4	0.0	.4	.2
Lys	3	3.6	3.0	3.0	2.9	3.5	3.0	2.9	2.9	3.1
Arg	6	5.0	5.0	5.4	5.4	5.6	5.4	5.5	5.5	5.4
(Trp)	0									

[a] Derived from nucleotide sequence published by Rotwein, et al., supra.

b. __IGF-1 purified from broth__
__of strain M+IMB206S1__

Amino acid composition analysis was performed on the
final IGF-1 purified from broth of strain M+IMB206S1 and
a concentration of 8.4 mg/ml was calculated. Table IV
shows the amino acid ratios estimated for the purified
IGF-1 and the actual published amino acid ratios for
human IGF-1. The estimated and published ratios are in
close agreement, and slight deviations in the estimated
and published compositions are within the expected limits
of this analysis.

TABLE IV

IGF-1 Purified from broth of Strain M+IMB206S1
Amino Acid Composition Analysis Data

Amino Acid	Published Composition[a]	Experimental
Asp (+Asn)	5	5.8
Thr	3	3.1
Ser	5	4.9
Glu (+Gln)	6	6.9
Pro	5	3.0
Gly	7	7.4
Ala	6	6.8
Cys	6	5.5
Val	3	2.6
Met	1	0.9
Ile	1	0.66
Leu	6	7.1
Tyr	3	2.1
Phe	4	3.6
His	0	0
Lys	3	3.3
Arg	6	6.7
Trp	0	

[a] Derived from nucleotide sequence published by Rotwein
et al. (supra).

-83-

5   **F. Protein Sequence Analysis**

        1. **IGF-1 Purified from broth f**
            strain G+IMB204814

        To determine the entire amino acid sequence of the

10 purified IGF-1, samples of this material were loaded

    directly onto an Applied Biosystems (Foster City, CA)

    470/120 Gas Phase Protein sequencer. Sequencing was

    performed according to the method described by

    Hunkapiller and Hood [Science 219:650 (1983)]. The

15 material was sequenced from the N-terminal amino acid

    through as much of the remainder of the protein sequence

    as possible. This analysis generated the sequence of the

    first 59 residues of the purified protein.

        Because the amino acid at residue 59 is a

20 methionine residue, and cyanogen bromide cleaves proteins

    after methionine residues, it was possible to isolate the

    peptide consisting of the C-terminal 11 amino acids

    (residues 60-70) of purified IGF-1 for use in completing

    the protein sequence analysis of the purified material.

25 The C-terminal 11 amino acids of the purified IGF-1 were

    obtained as a peptide fragment isolated from cyanogen

    bromide-treated IGF-1 by HPLC, using the same C4 column

    as described in Example 3A1. This fragment was loaded

    onto the protein sequencer to generate the sequence of

30 the C-terminal amino acids (amino acids 60-70).

        Every residue of the material purified

    according to the invention process has been positively

    identified except for the cysteines. These residues are

    identified by the complete absence of any residue in that

35 cycle. The entire sequence corresponded to authentic

    human IGF-1.

        b. **IGF-1 purified from broth of**
            strain M+IMB20681

40   Amino terminal sequence analysis of IGF-1 purified

    from broth f strain M+IMB206S1 revealed that the first

-84-

30 amino acids were identical to the N-terminal sequence
of human IGF-1.

     The invention has been described in detail with
respect to certain particular embodiments thereof, but
5   reasonable variations and modifications, within the
spirit and scope of the present disclosure, are
contemplated by the present disclosure and the appended
claims.

THAT WHICH IS CLAIMED IS:

    1. Method for the purification of monomeric, intact, correctly-folded insulin-like growth factor-1 peptide (IGF-1) from medium containing IGF-1 peptides, said method comprising:

    (a) contacting said medium with a sufficient quantity of first cation exchange material under conditions suitable to adsorb at least about 95% of total IGF-1 from said medium,

    (b) eluting the adsorbed IGF-1 from the IGF-1-containing cation exchange material of step (a) by contacting said cation exchange material with a sufficient quantity of a solvent system which has a sufficiently high pH or ionic strength so as to displace substantially all of said IGF-1 from said cation exchange material,

    (c) contacting the IGF-1-containing fractions of the eluate of step (b), in a suitable solvent system, with a sufficient quantity of a first hydrophobic interaction chromatography matrix under conditions suitable to adsorb in the range of about 95 up to 100% of said IGF-1 from said eluate,

    (d) eluting the adsorbed IGF-1 from said first hydrophobic interaction chromatography matrix by contacting said matrix first with in the range of about 1 up to 10 volumes, relative to the volume of matrix, of a buffer system having a sufficiently low conductivity so as to displace aberrant IGF-1 peptides from said matrix, without displacing significant quantities of the intact, monomeric, correctly-folded form of adsorbed IGF-1 from said first hydrophobic interaction chromatography matrix, followed by contacting said matrix with in the range of about 1 up to 10 volumes, relative to the volume of matrix, of a buffer system having an elevated pH, wherein said elevated pH is sufficiently high so as to displace substantially all of the remaining adsorbed forms of IGF-1 from said matrix,

(e)   contacting the fractions eluted according to step (d) employing said buffer system having an elevated pH which contain, as the predominant form of IGF-1, intact, monomeric, correctly-folded IGF-1, wherein said
5   contacting is carried out with a sufficient quantity of a second cation exchange matrix and under conditions suitable to adsorb in the range of about 95 up to 100% of total IGF-1 from said eluate,

(f)   eluting the adsorbed IGF-1 from said second
10   cation exchange matrix by contacting said matrix with at least 1 volume, relative to the volume of matrix, of a buffer system having a sufficient ionic strength so as to differentially displace substantially all of the IGF-1 peptides from said matrix,
15   (g)   contacting the intact, monomeric, correctly-folded IGF-1-containing fractions of the eluate from step (f), in a suitable solvent system, with either,

(1)   a sufficient quantity of a second hydrophobic interaction chromatography matrix under
20   conditions suitable to adsorb in the range of about 95 up to 100% of all forms of IGF-1 from said eluate, or,

(2)   a sufficient quantity of a gel filtration chromatography matrix having suitable pore size to effect resolution of the intact, monomeric correctly-folded form
25   of IGF-1 from substantially all multimeric forms of IGF-1, and

(h)(1)   after step (g)(1) eluting the adsorbed IGF-1 from said second hydrophobic interaction chromatography matrix by contacting said matrix with in the range of
30   about 1 up to 10 volumes, relative to the volume of matrix, of a buffer system having a sufficiently low conductivity so as to displace substantially all forms of IGF-1 other than the intact, monomeric, correctly-folded form of IGF-1 from said matrix, without displacing
35   significant quantities of the intact, monomeric, correctly-folded form of adsorbed IGF-1 from said second hydrophobic interaction chr matography matrix, followed

-87-

by contacting said matrix with in the range of about 1 up
to 10 volumes, relative t the volume of matrix, of a
buffer system having a sufficiently high pH so as to
displace substantially all of the remaining adsorbed IGF-
5   1 from said matrix or,

        (2) after step (g)(2) eluting said gel
filtration chromatography matrix with a sufficient
quantity of elution buffer so as to cause the intact,
monomeric correctly-folded form of IGF-1 to be resolved
10   from said multimeric forms of IGF-1.

        2. A method according to Claim 1 wherein said
medium contains at least 0.01 grams of IGF-1 peptides per
liter of said medium.

15

        3. A method according to Claim 1 wherein said
first cation exchange matrix is a strong cation exchange
matrix capable of having a high flow rate.

20   4. A method according to Claim 3 wherein said
first cation exchange matrix is selected from
carboxymethylated or sulfonated cation exchange media.

        5. A method according to Claim 4 wherein said
25   first cation exchange matrix is a sulfylpropylated
cellulose matrix.

        6. A method according to Claim 3 wherein the
contacting contemplated by step (a) is carried out at a
30   temperature in the range of about 2 up to 30°C.

        7. A method according to Claim 3 wherein the
contacting contemplated by step (a) is carried out by
passing the medium containing IGF-1 through a column
35   containing said first cation exchange material.

8. A method according t Claim 7 wherein, prior to step (b), said matrix is contacted with in the range of about one up to five column volumes, relative to the volume of matrix, of a dilute, weak acid solution,

5   followed by in the range of about zero up to six column volumes, relative to the volume of matrix, of a buffered solution of a dilute, weak acid having a pH of 5 and containing 0.2 M sodium chloride.

10   9. A method according to Claim 8 wherein the dilute, weak acid is an acetic acid or phosphoric acid solution.

10. A method according to Claim 9 wherein about

15 two volumes of said dilute, weak acid solution are employed; wherein said dilute, weak acid solution is a 0.02 M acetic acid solution; wherein about four volumes of said buffered solution having a pH of 5 and containing 0.2 M sodium chloride are employed; and wherein said

20 buffered solution is a 0.02 M sodium acetate solution having a pH of 5 and containing 0.2 M sodium chloride.

11. A method according to Claim 1 wherein suitable solvent systems for the step (b) elution are buffered

25 solutions of a weak, dilute acid having a pH of about 5.5 and containing about 1.0 M sodium chloride.

12. A method according to Claim 11 wherein, in the elution of IGF-1 from the first cation exchange matrix,

30 in the range of about 3 up to 10 volumes, per volume of said matrix, of said suitable solvent system is employed.

13. A method according to Claim 11 wherein said suitable solvent system for the step (b) elution is a

35 0.02 M sodium acetate solution having a pH of 5.5 and containing 1.0 M sodium chloride.

14. A method acc rding to Claim 13 wherein the IGF-1-containing eluate fractions of step (b) are treated, prior to step (c), by diluting said eluate with a sufficient volume of a buffered salt-containing
5   solution, pH 4.5, so as to render the salt concentration of the diluted eluate in the range of about 0.2 up to 2 $\underline{M}$, and the pH of the diluted eluate about 4.5.

15. A method according to Claim 1 wherein the
10   contacting contemplated by step (c) is carried out by passing the IGF-1-containing fractions of the eluate of step (b) through a column containing said first hydrophobic interaction chromatography matrix; wherein the contacting contemplated by step (g)(1) is carried out
15   by passing the IGF-1-containing fractions of the eluate of step (f) through a column containing said second hydrophobic interaction chromatography matrix; and wherein said first and said second hydrophobic interaction chromatography matrices are each
20   independently selected from an alkyl- or aryl-substituted hydrophobic interaction chromatography matrix.

16. A method according to Claim 15 wherein said hydrophobic interaction chromatography matrix is a
25   butyl-, octyl-, or phenyl-substituted hydrophobic interaction chromatography matrix.

17. A method according to Claim 16 wherein said hydrophobic interaction chromatography matrix is a butyl-
30   substituted, poly(methacrylate)-supported HIC matrix.

18. A method according to Claim 15 wherein the contacting contemplated by steps (c) and (g)(1) are each independently carried out at a temperature in the range
35   of about 15 up to 30°C.

19. A method according to Claim 15 wherein said first and/or second hydrophobic interaction chromatography matrix is a non-silica-based matrix, and is regenerated by four sequential washes comprising:

5   in the range of 3-10 column volumes of water, in the range of 3-10 column volumes of a 0.5 $\underline{N}$ sodium hydroxide solution,

           in the range of 3-10 column volumes of a 50% aqueous methanol solution, and finally

10   in the range of 3-10 column volumes of water, and thereafter, the column was equilibrated with in the range of 5-10 column volumes of an ammonium sulfate-containing acetate/phosphate buffer having a pH of 4.5.

15   20. A method according to Claim 1 wherein the elution of adsorbed IGF-1 from the IGF-1-containing matrix according to steps (d) and (h)(1) comprises, in each step, contacting said matrix

        (i)   first with a linear salt gradient of a

20   buffered solution having a pH of about 4.5 in a quantity sufficient to produce a substantially salt-free eluate, then

        (ii)   with a linear gradient of a substantially salt-free buffered solution having an

25   initial pH of about 4.5 in a quantity sufficient to raise the pH of said eluate up to about 6.5.

21. A method according to Claim 1 wherein a

30 portion of the eluate of step (d) eluted at elevated pH is again subjected to steps (c) and (d), and fractions of the eluates of both step (d) elutions which contain as the predominant form of IGF-1 intact, monomeric, correctly-folded IGF-1 are combined before proceeding

35 with step (e).

22. A method acc rding to Claim 1 wherein the
contacting contemplated by step (e) is carried out by
passing said fractions containing IGF-1 through a column
containing said second cation exchange material; and

5   wherein said second cation exchange matrix is a strong
cation exchange matrix capable of high resolution.

23. A method according to Claim 22 wherein the pH
of the eluate of step (d) is adjusted to about 4.5, and

10   said eluate is diluted with at least 1 volume of water or
a low conductivity buffer prior to the contacting
contemplated by step (e).

24. A method according to Claim 22 wherein said

15   second cation exchange matrix is selected from
carboxymethylated or sulfonated cation exchange media.

25. A method according to Claim 24 wherein said
second cation exchange matrix is a sulfylmethyl agarose.

20

26. A method according to Claim 22 wherein the
contacting contemplated by step (e) is carried out at a
temperature in the range of about 2 up to 30°C.

25   27.   A method according to Claim 22 wherein
said second cation exchange matrix is
activated/regenerated by four sequential washes
comprising:
          in the range of 3-10 column volumes of water,

30   in the range of 3-10 column volumes of a 0.5N
sodium hydroxide solution,
          in the range of 3-10 column volumes of a 50%
aqueous methanol solution, and finally
          in the range of 3-10 column volumes of water,

35   and thereafter, the column was equilibrated with
          in the range f 3-5 column volumes of 0.5M
sodium acetate, pH 4.5, and

-92-

in the range of 10-20 column volumes of 0.05M sodium acetate, pH 4.5.

28. A method according to Claim 22 wherein, prior to step (f), said second cation exchange matrix is washed by contacting it with in the range of about 1 up to 5 volumes, relative to the volume of matrix, of at least one solution which is a dilute buffer of a weak acid.

29. A method according to Claim 28 wherein said second cation exchange matrix is washed with at least one solution which is an acetic acid or phosphoric acid based buffer solution.

30. A method according to Claim 29 wherein said first and/or second cation exchange matrix is washed by contacting it with a solution(s) selected from the group consisting of (1) a buffer solution which comprises a 0.05 M sodium acetate solution and has a pH of 4.5; and (2) a buffer solution which comprises a 0.05 M sodium acetate solution and has a pH of about 4.5, followed by a buffer solution which comprises a 0.05 M sodium acetate solution and has a pH of about 5.5.

31. A method according to Claim 1 wherein suitable solvent systems for the step (f) elution comprise a sodium chloride gradient in a sodium acetate buffer, pH 5.5.

32. A method according to Claim 31 wherein, in the elution of IGF-1 from the second cation exchange matrix, in the range of about 3 up to 15 volumes, per volume of said matrix, of said suitable solvent system is employed.

33. A m thod according to Claim 32 wherein said sodium chloride gradient is provided by combining, as a

linear gradient, a first solvent system and a second
solvent system;

wherein said first solvent system comprises a
0.05M sodium acetate solution, pH 5.5, and

5   wherein said second solvent system comprises a
0.05M sodium acetate/0.3M sodium chloride solution, pH
5.5.

34.   A method according to Claim 1 wherein the IGF-
10   1-containing eluate of step (f) is treated, prior to step
(g)(1), by diluting said eluate with a sufficient volume
of a buffered salt-containing solution, pH 4.5, so as to
render the salt concentration of the diluted eluate in
the range of about 0.2 up to 2 M, and the pH of the
15   diluted eluate about 4.5.

35.   A method according to Claim 15 wherein said
second hydrophobic interaction chromatography matrix is a
non-silica based matrix, and is regenerated by four
20   sequential washes comprising:

in the range of 3-10 column volumes of water,

in the range of 3-10 column volumes of a 0.5N
sodium hydroxide solution,

in the range of 3-10 column volumes of a 50%
25   aqueous methanol solution, and finally

in the range of 3-10 column volumes of water,
and thereafter, the column was equilibrated with in the
range of 5-10 column volumes of an ammonium sulfate-
containing acetate/phosphate buffer having a pH of 4.5.

30
36.   A method according to Claim 1 wherein a
portion of the eluate of step (h)(1) eluted at elevated
pH is again subjected to steps (g)(1) and (h)(1) and
fractions of the eluates of both step (h)(1) elutions
35   which contain as the predominant form of IGF-1 intact,

-94-

monomeric, correctly-folded IGF-1 are combined and
retained.

5   37.   Method for the purification of monomeric,
intact, correctly-folded insulin-like growth factor-1
(IGF-1) peptide from medium containing IGF-1 peptides,
wherein said medium containing IGF-1 is the substantially
cell-free fermentation broth from a high cell density
yeast fermentation operation, and wherein said yeast are
transformed with at least one DNA fragment comprising, in
10   the direction of transcription, the following DNA
sequences:
          (i)   a promoter region of a methanol-responsive gene
of Pichia pastoris,
          (ii) a DNA sequence encoding a polypeptide
15   consisting of:
                    (a)   the S. cerevisiae alpha mating factor pre-
                           pro sequence, including a processing site
                           selected from the group consisting of lys-
                           arg and lys-arg-(glu-ala)$_x$, wherein x is an
20   integer between 1 and about 3; and
                    (b)   an insulin-like growth factor-1 (IGF-1)
                           peptide; and
          (iii) a transcription terminator functional in
P. pastoris,
25   wherein said DNA sequences are operationally associated
with one another for transcription of the sequences
encoding said polypeptide, said method comprising:
          (a) optionally, diluting said IGF-1-containing
medium with a low conductivity buffered medium having the
30   same pH as the media used to equilibrate the cation
exchange matrix employed in step (b) below,
          (b) contacting said medium with a sufficient
quantity of a sulfylpropylated cation exchange media
under conditions suitable to adsorb at least about 95% of
35   said IGF-1 from said medium; wherein at least 0.05
liters, per gram of IGF-1 in said medium, of said cation

-95-

exchange material are employed; and wherein said
contacting is carried out at a temperature in the range
of about 2 up to 30°C,

(c) contacting the IGF-1-containing cation

5   exchange material with at least about 2 volumes, per
volume of said cation exchange material, of a 0.02 $\underline{M}$
acetic acid solution, followed by about four volumes of a
0.02 M sodium acetate solution having a pH of 5 and
containing 0.2 M sodium chloride,

10   (d) eluting the adsorbed IGF-1 from said IGF-1-
containing cation exchange matrix material of step (c) by
contacting said matrix material with a sufficient
quantity of a solvent system comprising a 0.02 M sodium
acetate solution having a pH of 5.5 and containing 1.0 M

15   sodium chloride,

(e) contacting the IGF-1-containing eluate of
step (d) with a sufficient volume of a buffered ammonium
sulfate-containing solution, pH 4.5, so as to render the
ammonium sulfate concentration of the eluate in the range

20   of about 0.4 up to 0.8 $\underline{M}$, and the pH of the diluted
eluate about 4.5,

(f) contacting the product of step (e) with a
sufficient quantity of a first hydrophobic interaction
chromatography matrix under conditions suitable to adsorb

25   in the range of about 95 up to 100% of said IGF-1 from
said eluate, wherein said first hydrophobic interaction
chromatography matrix is a butyl-substituted,
poly(methacrylate)-supported hydrophobic interaction
chromatography matrix; wherein at least about 0.05

30   liters, per gram of IGF-1 in said medium, of said first
hydrophobic interaction chromatography matrix are
employed; and wherein said contacting is carried out at a
temperature in the range of about 20 up to 25°C,

(g) eluting the adsorbed IGF-1 from said first

35   hydrophobic interaction chromatography matrix by
contacting said matrix:

-96-

(1)   first with a quantity of a linear salt gradient
       of a buffered solution having a pH of about 4.5
       sufficient to produce a substantially ammonium
       sulfate-free eluate, then

5   (2)   with a quantity of a linear gradient of a
       substantially ammonium sulfate-free buffered
       solution having an initial pH of about 4.5
       sufficient to raise the pH of said eluate up to
       about 6.5,

10   (h)   contacting at least a portion of the
eluate fractions obtained in step (g)(2) with a
sufficient additional quantity of said first hydrophobic
interaction chromatography matrix under conditions
suitable to adsorb in the range of about 95 up to 100% of

15 residual quantities of IGF-1 from said eluate; wherein at
least about 0.05 liters, per gram of IGF-1 in said
medium, of said first hydrophobic interaction
chromatography resin are employed; and wherein said
contacting is carried out at a temperature in the range

20 of about 20 up to 25°C.

(i)   eluting the adsorbed IGF-1 from said first
hydrophobic interaction chromatography matrix by
contacting said matrix:

(1)   first with a quantity of a linear salt gradient
       of a buffered solution having a pH of about 4.5
25   sufficient to produce a substantially ammonium
       sulfate-free eluate, then

(2)   with a quantity of a linear gradient of a
       substantially ammonium sulfate-free buffered
30   solution having an initial pH of about 4.5
       sufficient to raise the pH of said eluate up to
       about 6.5,

(j) contacting those portions of the combined
eluate from steps (g)(2) and (i)(2) which contain, as the
35 predominant form of IGF-1, intact, monomeric, correctly-
folded IGF-1, wherein said contacting is carried out with

-97-

a sufficient quantity of a second cation exchange matrix
material and under conditions suitable to adsorb in the
range of about 95 up to 100% of said IGF-1 from said
eluate; wherein said matrix comprises a sulfylmethylated

5   agarose matrix; wherein at least 0.05 liters, per gram of
IGF-1 in said medium, of said cation exchange material
are employed; and wherein said contacting is carried out
at a temperature in the range of about 2 up to 30°C,

　　　　　　　(k) contacting the IGF-1-containing second
10   cation exchange matrix material with at least one, up to
about five volumes, per volume of said cation exchange
material, of a 0.05 $\underline{M}$ sodium acetate solution, pH 4.5,

　　　　　　　(l) eluting the adsorbed IGF-1 from said second
cation exchange matrix material by contacting said matrix
15   material with at least five volumes, relative to the
volume of matrix, of a sodium chloride gradient, which is
provided by combining, as a linear gradient, a first
solvent system and a second solvent system;

　　　　　　　wherein said first solvent system comprises a
20   0.05 $\underline{M}$ sodium acetate solution, pH 5.5, and

　　　　　　　wherein said second solvent system comprises a
0.05 $\underline{M}$ sodium acetate/0.3 $\underline{M}$ sodium chloride solution, pH
5.5,

　　　　　　　(m) either
25   　　　　　　　(1) diluting the intact, monomeric, correctly-
folded IGF-1 containing fractions of the eluate of step
(l) with at least one volume of a buffered ammonium
sulfate-containing solution, pH 4.5, so as to render the
ammonium sulfate concentration of the eluate in the range
30   of about 0.2 up to 2.0 $\underline{M}$ and the pH of the diluted eluate
about 4.5, and contacting the diluted eluate with a
sufficient quantity of a second hydrophobic interaction
chromatography matrix under conditions suitable to adsorb
in the range of about 95 up to 100% of said IGF-1 from
35   said eluate; wherein said s cond hydrophobic interaction
chromatography matrix is a butyl-substituted hydrophobic

interaction chromatography matrix; wherein at least about
0.05 liters, per gram of IGF-1 in said medium, of said
second hydrophobic interaction chromatography matrix are
employed; and wherein said contacting is carried out at a
5   temperature in the range of about 20 up to 25°C or,

(2) contacting the intact, monomeric,
correctly-folded IGF-1-containing fractions of the eluate
from step (1) with a sufficient quantity of a gel
filtration chromatography matrix having suitable pore
10   size to effect resolution of the intact, monomeric
correctly-folded form of IGF-1 from substantially all
multimeric forms of IGF-1, and

(n)(1) after step (m)(1) eluting the adsorbed
IGF-1 from said second hydrophobic interaction
15   chromatography matrix by contacting said matrix:

(i)   first with a quantity of a linear salt gradient
of a buffered solution having a pH of about 4.5
sufficient to produce a substantially ammonium
sulfate-free eluate, then

20   (ii)   with a quantity of a linear gradient of a
substantially ammonium sulfate-free buffered
solution having an initial pH of about 4.5
sufficient to raise the pH of said eluate up to
about 6.5, or

25   (2) after step (m)(2) eluting said gel
filtration chromatography matrix with a sufficient
quantity of elution buffer so as to cause the intact,
monomeric correctly-folded form of IGF-1 to be resolved
from said multimeric forms of IGF-1.

30

38. A method according to Claim 37 wherein a
portion of the eluate of step (n)(1) eluted at elevated
pH is again contacted with said hydrophobic interaction
chromatography matrix and eluted according to step
35   (n)(1), and fractions of the eluates f both step (n)(1)
elutions which contain as the predominant form f IGF-1

-99-

intact, monomeric, correctly-folded IGF-1 are combined and retained.

39. Method for the purification of monomeric, intact, correctly-folded insulin-like growth factor-1 peptide (IGF-1) from medium containing IGF-1 peptides, said method comprising:

(a) contacting said medium with a sufficient quantity of first cation exchange material under conditions suitable to adsorb at least about 95% of total IGF-1 from said medium,

(b) eluting the adsorbed IGF-1 from the IGF-1-containing cation exchange material of step (a) by contacting said cation exchange material with a sufficient quantity of a solvent system which has a sufficiently high pH or ionic strength so as to displace substantially all of said IGF-1 from said cation exchange material,

(c) contacting the IGF-1-containing fractions of the eluate of step (b), in a suitable solvent system, with a sufficient quantity of a first hydrophobic interaction chromatography matrix under conditions suitable to adsorb in the range of about 95 up to 100% of said IGF-1 from said eluate,

(d) eluting the adsorbed IGF-1 from said first hydrophobic interaction chromatography matrix by contacting said matrix first with in the range of about 1 up to 10 volumes, relative to the volume of matrix, of a buffer system having a sufficiently low conductivity so as to displace aberrant IGF-1 peptides from said matrix, without displacing significant quantities of the intact, monomeric, correctly-folded form of adsorbed IGF-1 from said first hydrophobic interaction chromatography matrix, followed by contacting said matrix with in the range of about 1 up to 10 volumes, relative to the volume of matrix, of a buffer system having an elevated pH, wherein

said elevated pH is sufficiently high so as to displace substantially all of the remaining adsorbed forms of IGF-1 from said matrix.

5   40.   A method according to Claim 39 wherein prior to the elution in step (b) the IGF-1-containing cation exchange chromatography matrix is subjected to a step-wise wash system selected from the group consisting of: (1)(i) a dilute acetic acid solution followed by (ii)
10   an acetate buffer solution having a pH of about 5 and having a concentration of about 0.2 M salt; (2)(i) a dilute acetic acid solution followed by (ii) an acetate buffered solution having a pH of about 5, followed by (iii) an acetate buffered solution having a pH of about
15   5.5.

       41.   A method according to Claim 39 wherein prior to the elution in step (b) the IGF-1-containing cation exchange chromatography matrix is subjected to a step-
20   wise wash system selected from the group consisting of: (1) 20 mM acetic acid followed by 20 mM sodium acetate, pH 5, containing 0.2 M NaCl or (2) 20 mM acetic acid followed by 50 mM sodium acetate, pH 5, followed by 50 mM sodium acetate, pH 5.5.

25
       42.   A method according to Claim 39 wherein prior to the elution in step (b) the IGF-1-containing cation exchange chromatography matrix is subjected to a step-wise wash system consisting of: (1) a dilute acetic
30   acid solution; (2) a salt-free acetate buffer solution having a pH of about 5.5; (3) an acetate buffer solution having a pH of about 5.5 and having about 0.05 M salt; and (4) an acetate buffer solution having a pH of about 5.5 and having about 0.1 M salt.

35

43. A method according to Claim 42 wherein the step-wise wash system consists of 20 mM acetic acid followed by 50 mM sodium acetate, pH 5.5, followed by 0.05 M NaCl in 50 mM sodium acetate, pH 5.5, followed by
5   0.1 M NaCl in 50 mM sodium acetate, pH 5.5.

44. A method according to Claim 42 wherein the solvent system of step (b) is an acetate buffer solution having a pH of about 5.5 and having about 0.3 M salt.

10

45. A method according to Claim 43 wherein the solvent system of step (b) is 0.3 M NaCl, 50 mM sodium acetate, pH 5.5.

15   46. A method according to Claim 39 wherein the solvent system in step (b) is a linear salt gradient starting with a substantially salt-free buffer solution having a pH of about 5.5 and ending with a buffer solution having substantially the same pH and having a
20   salt concentration of about 0.5 M.

47. A method according to Claim 46 wherein the salt is sodium chloride.

25   48. A method according to Claim 39 wherein the buffer system having a sufficiently low conductivity is a linear gradient starting at 20% saturated ammonium sulfate buffered at pH 4.5 with 50 mM sodium acetate/phosphate, and ending with 0% ammonium sulfate
30   buffered at pH 4.5 with the same buffer.

49. A method according to Claim 48 wherein the buffer system having an elevated pH is an increasing pH gradient starting at a pH of about 4.5 and ending at a pH
35   of about 6.5.

50. A method according to Claim 39 wherein
the buffer system having a sufficiently low conductivity
is a linear gradient starting at 20% saturated ammonium
sulfate buffered at pH 5.0 with 50 mM sodium
5   acetate/phosphate, and ending with 0% ammonium sulfate
buffered at pH 4.0 with the same buffer.

51. A method according to Claim 50 wherein
after the linear gradient, the hydrophobic interaction
10 chromatography matrix is further contacted with an
ammonium sulfate-free solution buffered at pH 4.0.

52. A method according to Claim 51 wherein
the buffer system having an elevated pH is a solution
15 having a pH in the range of 6.5 to 7.5.

53. A method according to Claim 39, said
method further comprising:
        (e) after step (d), contacting the intact,
20 monomeric, correctly-folded IGF-1-containing fractions of
the eluate from step (d) obtained after contacting said
matrix with a buffer system having an elevated pH, in a
suitable solvent system, with a sufficient quantity of a
gel filtration chromatography matrix having suitable pore
25 size to effect resolution of the intact, monomeric
correctly-folded form of IGF-1 from substantially all
multimeric forms of IGF-1; and
        (f) eluting said gel filtration chromatography
matrix with a sufficient quantity of an eluent so as to
30 cause the intact, monomeric correctly-folded form of IGF-
1 to be resolved from said multimeric forms of IGF-1.

54. A method according to Claim 53 wherein
said eluent is a solution having a concentration of about
35 50 mM ammonium acetate and a pH of about 6.0.

55.   A method according to Claim 53 wherein the
gel filtration chromatography matrix is a polymer-based
resin, and the eluent in step (f) is an acetic acid
solution.

5

56.   A method according to Claim 55 wherein the
gel filtration chromatography matrix is Toyopearl HW50F
and the eluent is an acetic acid solution having a
concentration of about 0.2 M.

10   57.   A method for the purification of
monomeric, intact, correctly-folded insulin-like growth
factor-1 peptide (IGF-1) from medium containing IGF-1
peptides, said method comprising:
          (a)   contacting said medium with a sufficient
15   quantity of first cation exchange material under
conditions suitable to adsorb at least about 95% of total
IGF-1 from said medium;
          (b)   eluting the adsorbed IGF-1 from the
IGF-1-containing cation exchange material of step (a) by
20   contacting said cation exchange material with a
sufficient quantity of a buffered 0.3 M NaCl solution
having a pH of about 5.5 so as to displace substantially
all of said IGF-1 from said cation exchange material,
wherein prior to said elution the IGF-1-containing cation
25   exchange chromatography matrix is subjected to a step-
wise wash system consisting of: (1) a dilute acetic acid
solution; (2) a salt-free buffer solution having a pH of
about 5.5; and (3) an buffer solution having a pH of
about 5.5 and having about 0.1 M salt;
30   (c)   contacting the IGF-1-containing fractions
of the eluate of step (b), in a suitable solvent system,
with a sufficient quantity of a first hydrophobic
interaction chromatography matrix under conditions
suitable to adsorb in the range of about 95 up to 100% of
35   said IGF-1 from said eluate;

-104-

(d)   eluting the adsorbed IGF-1 from said first
hydrophobic interaction chromatography matrix by
contacting said matrix first with in the range of about 1
up to 10 volumes, relative to the volume of matrix, of a
5   low conductivity buffer system which is a linear gradient
starting at 20% saturated ammonium sulfate buffered at pH
5.0 and ending with 0% ammonium sulfate buffered at pH
4.0 with the same buffer, whereby aberrant IGF-1 peptides
are displaced from said matrix, without displacing
10   significant quantities of the intact, monomeric,
correctly-folded form of adsorbed IGF-1 from said first
hydrophobic interaction chromatography matrix, followed
by contacting said matrix with in the range of about 1 up
to 10 volumes, relative to the volume of matrix, of an
15   elevated pH buffer system which is an increasing pH
gradient starting at about pH 4 and ending at about pH 7,
whereby substantially all of the remaining adsorbed forms
of IGF-1 are displaced from said matrix;
(e)   contacting the fractions of the eluate of step
20   (d) which contain, as the predominant form of IGF-1,
intact, monomeric, correctly-folded IGF-1, in a solution
suitable to adjust the pH of said fractions to about pH
4.5, with a sufficient quantity of a second cation
exchange matrix and under conditions suitable to adsorb
25   in the range of about 95 up to 100% of total IGF-1 from
said eluate;
(f)   eluting the adsorbed IGF-1 from said second
cation exchange matrix by contacting said matrix with at
least 1 volume, relative to the volume of matrix, of a
30   buffer system which is a linear salt gradient starting at
0% NaCl buffered at pH 5.5, and ending with 0.3 M NaCl
buffered at pH 5.5 with the same buffer, whereby
substantially all of the IGF-1 peptides are
differentially displaced from said matrix, and wherein,
35   prior to elution, said second cation exchange matrix is
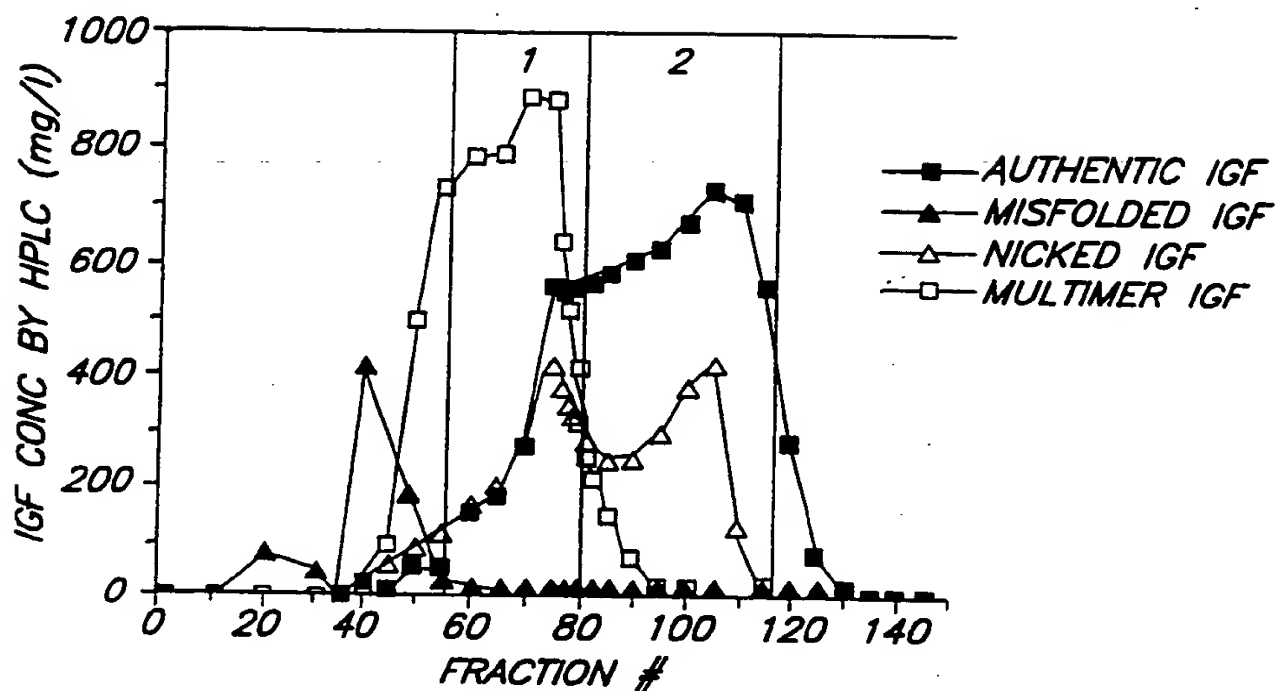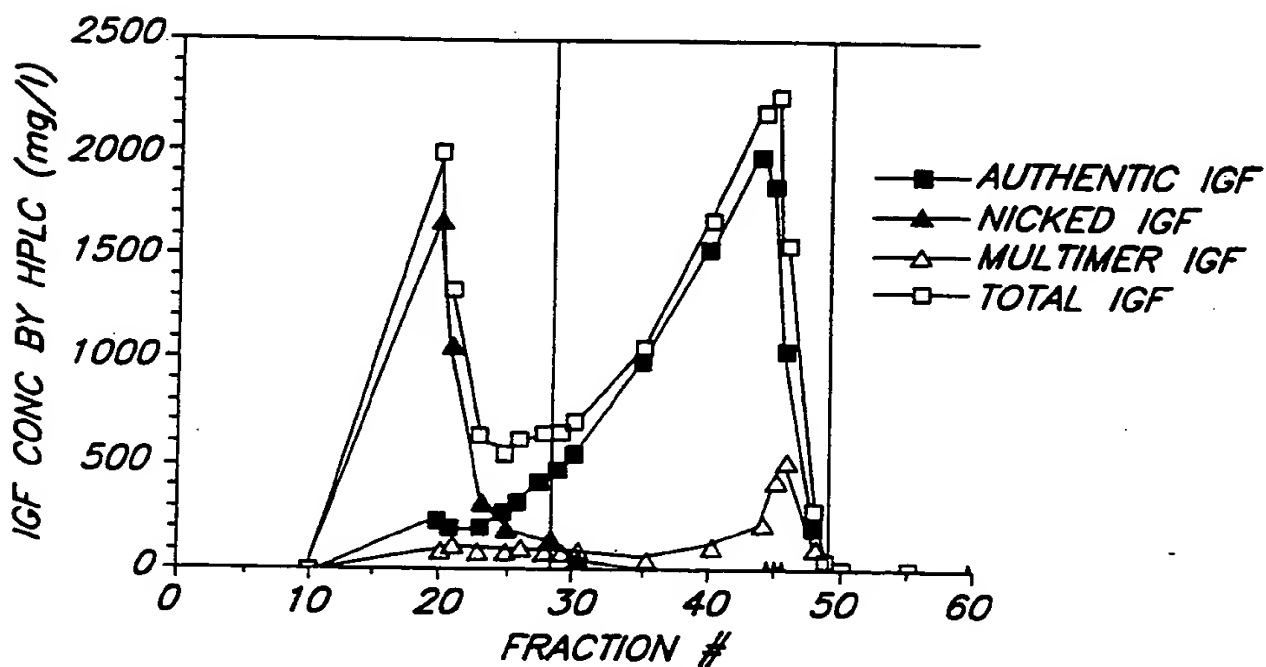washed by contacting said matrix with a sufficient

quantity of a buffered, salt-free solution having a pH of
about pH 4.5, followed by a sufficient quantity of a
buffered, salt-free solution having a pH of about pH 5.5;

5   (g) after step (f), contacting the intact,
monomeric, correctly-folded IGF-1-containing fractions of
the eluate from step (f), in a suitable solvent system,
with a sufficient quantity of Toyopearl HW50F gel
filtration chromatography matrix so as to effect
10   resolution of the intact, monomeric correctly-folded form
of IGF-1 from substantially all multimeric forms of IGF-
1; and

      (h) eluting said gel filtration chromatography
matrix with a sufficient quantity of 0.2 M acetic acid
15   eluent so as to cause the intact, monomeric correctly-
folded form of IGF-1 to be resolved from said multimeric
forms of IGF-1.

FIG. 1

FIG. 2

I. CLASSIFICATION OF SUBJECT MATTER   (if several classification symbols apply, indicate all)*

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K1/14;

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT*

Category*	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	EP,A,0 360 411 (KABI-VITRUM) 28 March 1990 see column 4	39-52
Y	DOMESTIC ANIMAL ENDOCRINOLOGY vol. 7, no. 3, 1990, AUBURN, U.S.A. pages 383 - 393; ARMSTRONG ET AL: 'Biological activity of insulin-like growth factor-1 purified from chicken serum' page 383 (Abstract) page 387	39-52

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* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 APRIL 1992	2 3 APR 1992

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	KORSNER S.E.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT	(CONTINUED FROM THE SECOND SHEET)	
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CHEMICAL ABSTRACTS, vol. 107, 1987, Columbus, Ohio, US; abstract no. 33292, BAXTER ET AL: 'Natural and recombinant DNA-derived human insulin-like growth factor -1 compared for use in radioligand assays' page 86 ; see abstract & CLINICAL CHEMISTRY vol. 33, no. 4, 1987, pages 544 - 548;	1-57
A	JOURNAL OF ENDOCRINOLOGY vol. 128, no. 1, January 1991, LETCHWORTH, ENGLAND pages 161 - 168; WANG ET AL: 'Purification and assay of insulin-like growth factor-binding protein-1: measurement of circulating levels throughout pregnancy' page 161 (abstract)	1-57

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 16/04/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0360411	28-03-90	AU-A- 4051889	23-03-90
		WO-A- 9002198	08-03-90
		EP-A- 0429502	05-06-91

FIG. 1

FIG. 2

IOCID: <WO___9212993A1TI_>